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Immunoglobulin, somatic hypermutation and the survival of B cells

Pedro José Ponce de Leão de Azevedo Geraldes

Orientadora: Marilia Cascalho, MD, PhD

Co-Orientador: Rui Victorino, MD, PhD.

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Sumário

O objetivo desta dissertação foi descobrir mecanismos de sobrevivência das células B durante o processo de resposta a antigénios. Este trabalho revelou que expressão da cadeia pesada da imunoglobulina é suficiente para assegurar a sobrevivência de linfócitos B na ausência de cadeias leves. Estes resultados sugerem que a cadeia pesada origina sinais de sobrevivência de forma constitutiva e independente de ligação a ligandos específicos para o receptor composto de cadeias de imunoglobulina pesadas e leves. Na segunda parte de tese, procurámos determinar mecanismos de sobrevivência de células B sujeitas a quebras do ADN durante os processos de mudança de isótipo e a hipermutação somática da imunoglobulina. Os nossos resultados revelaram que a enzima "activation induced cytidine deaminase" (AID), que inicia os processos de mudança de isótipo e de introdução de mutações somáticas nos genes codificadores das cadeias de anticorpos, (Muramatsu et al., 2000), é também responsável pela reparação do ADN, permitindo assim às células B evitar a apoptose induzida por danos persistentes no ADN. Observámos, (Wu et al., 2005), que a AID recruta a subunidade catalítica da ADN proteína quinase (DNA-PKcs) sugerindo que a AID coordena a reparação de quebras no ADN, contribuindo desta forma para a sobrevivência das células B.

Palavras chave

IgL repressible mouse
Cadeia pesada da Imunoglobulina
Sobrevivência de linfócitos B
Receptor das células B
Resposta a proteínas não enroladas
Citidina desaminase induzida pela ativação
ADN proteína quinase
Hipermutação somática
Mudança de isótipo
Reparação de quebras no ADN

Sumário alargado

A imunidade adaptativa é assim designada por não ser antecipatória, ao contrário da imunidade inata, desenvolvendo-se sempre em resposta a estímulos sejam estes infecciosos, tóxicos ou outros. Se, por um lado, a natureza não antecipatória da imunidade adaptativa limita o desenvolvimento rápido de respostas protetoras aquando do primeiro encontro com um novo estímulo imunológico, por outro, tem a capacidade de formar um histórico ou "imprint" da resposta a cada estímulo. Esta é a sua principal vantagem, na medida em que assegura uma proteção mais rápida e eficaz aquando de um segundo encontro com um mesmo estímulo. Este histórico e a sua manifestação são conhecidos por memória imunológica.

O advento da memória imunológica requer um reconhecimento específico do estímulo e a sobrevivência das células que medeiam a resposta a este primeiro encontro, de forma a permitir uma subsequente ativação e resposta efetora com maior rapidez e intensidade que a resposta inicial.

A memória imunológica foi historicamente a primeira característica da imunidade adaptativa a ser reconhecida (Panum, 1847). Apesar de largamente manipulada na prevenção de doenças, os mecanismos necessários para o desenvolvimento e manutenção da memória imunológica não são ainda inteiramente conhecidos. No início do século XX Von Behring e Kitasato (Silverstein, 1999) descobriram antitoxinas, demonstrando que algumas propriedades da imunidade adaptativa podiam ser transferidas pelo soro. Ehrlich (Ehrlich and Morgenroth, 1957) chamou aos efetores humorais anticorpos, e demonstrou que os estes criavam respostas imunes específicas. Landsteiner and Chase (Landsteiner and Chase, 1941) descobriram em 1941 que a imunidade podia ser transferida por células. Assim em meados do século XX os efetores da imunidade adaptativa foram reconhecidos (células e anticorpos) assim como as suas propriedades fundamentais (imunidade adquirida, especificidade e memória).

É geralmente aceite que o primeiro encontro com o antigénio estimula as células B (as células que produzem anticorpos) não só a diferenciarem-se em plasmócitos mas também a originarem populações de células de memória (Gray, 1988) e de plasmócitos de maior longevidade (Manz et al., 2002). As células B de memória diferem de células B "naives" na sua longevidade, no tipo de imunoglobulina (normalmente de isótopos diferentes) e na presença de mutações acumuladas nos exões variáveis (V) das cadeias pesadas e leves das imunoglobulinas, evidência de hipermutação somática (Meffre et al., 2001). A rapidez das respostas de memória deve-se não só a um maior número de células específicas para antigénio que o número de células "naives" com essa mesma especificidade (Ahmed and Gray, 1996), como também à maior afinidade do receptor B para o antigénio capaz de ser ativado por doses diminutas de antigénio, bem como devido a propriedades facilitadoras de ativação distintas de certas classes de IgG (Liu et al., 2010; Martin and Goodnow, 2002). As células B de memória que persistem, ou continuamente geradas por persistente presença de antigénio, são responsáveis pelas respostas aceleradas de eficácia aumentada que caracteriza as respostas de anticorpos secundárias. Uma vez que os plasmócitos não expressam Ig de superfície, e não se dividem, não contribuem para as propriedades das respostas secundárias, mas apenas para a persistência de anticorpos específicos que secretam enquanto vivem.

Desconhecem-se os mecanismos pelos quais as células B adquirem e mantêm longevidade. Lam et al. (Lam et al., 1997) mostraram que deleção do exão V da cadeia pesada, abolindo a expressão da cadeia pesada, causa a apoptose de células B maduras. Kraus, M. et al. (Kraus et al., 2004) demonstraram que inibição de sinal do receptor B em células B maduras conduz à morte rápida destas células (em dias), sugerindo que os sinais do receptor das células B são necessários para a sobrevivência destas células. Não ficou porém explicado se o receptor das células B promove a sobrevivência de células B através da ligação a um ligando genérico ou através de um sinal constitutivo independente de um ligando.

O objetivo desta dissertação foi assim descobrir os elementos que contribuem para a sobrevivência das células B durante o processo de ativação. Em primeiro lugar, questionamos se a sobrevivência das células B dependia da presença de um receptor B intacto. Apesar do trabalho de Kraus et al. (Kraus et al., 2004) evidenciar que a sobrevivência das células B depende de um sinal a partir do receptor B, observamos na natureza em diversas circunstâncias a existência de células B sem este receptor. Por exemplo, células B sem receptor que expressam um receptor do vírus Epstein Barr conseguem sobreviver (Casola et al., 2004), ou no caso dos camelos (Muyldermans and Lauwereys, 1999) e alguns tubarões (Greenberg et al., 1995a) em que as células B sobrevivem sem um receptor B convencional e secretam anticorpos incompletos apenas com a cadeia pesada da imunoglobulina.

No sentido de determinar se a cadeia pesada da imunoglobulina permite, por si só, a sobrevivência de células B, utilizamos um modelo em que a expressão da cadeia leve da imunoglobulina (IgL) podia ser transientemente reprimida: o "IgL repressible mouse". Este modelo permitiu estudar como a repressão da cadeia leve em células B que mantinham a expressão da cadeia pesada afetava a sua longevidade. As nossas observações indicam que células B que expressam apenas a cadeia pesada da imunoglobulina sobrevivem e persistem. Concluímos assim que a sobrevivência das células B não requer a expressão de um receptor B completo. Interessava agora elucidar o mecanismo responsável por esta observação. Uma vez que os nossos resultados demonstravam que as células B sem IgL expressam na sua superfície uma cadeia pesada integrada num receptor incompleto, propusemo-nos investigar a hipótese de que este receptor ser capaz de sinalizar. As nossas observações revelaram que é efetivamente o caso, o que nos levou a concluir que o sinal transduzido pela cadeia pesada pode ser o único mecanismo necessário para a sobrevivência destas células. Por outro lado, a acumulação de cadeias pesadas no retículo endoplasmático resultante da ausência de cadeias leves, também pode contribuir para a sua sobrevivência. De acordo com esta possibilidade, observámos que as células B IgL negativas exibiam ativação de uma resposta de stress à acumulação de proteínas no retículo endoplasmático: "unfolded protein response". Esta resposta de stress é responsável pela ativação de diversos fatores de sobrevivência em células B. Concluímos assim que as cadeias pesadas não emparelhadas com cadeias leves podem

promover a sobrevivência das células B ou através de sinais provenientes de complexos membranares incompletos ou através da ativação da "unfolded protein response" (Geraldes et al., 2007)

O surgimento de células B de grande longevidade após ativação é ainda mais intrigante considerando que a maioria é sujeita a numerosas quebras da cadeia dupla de ADN, durante a ativação e multiplicação clonal que acompanham a recombinação para mudança de isótipo e a hipermutação somática. Normalmente, as células respondem a quebras na cadeia dupla de ADN parando o ciclo celular de forma a terem tempo para reparar os danos (Nyberg et al., 2002) e respondem a danos persistentes induzindo apoptose, presumivelmente para evitar recombinação ilegítima (Nyberg et al., 2002).

O processo através do qual as células B de memória, que exibem isótopos alterados e mutações nos exões variáveis da imunoglobulina, sobrevivem a múltiplas quebras no ADN não e ainda claro. Procurámos assim determinar se a enzima "activation induced cytidine deaminase" (AID), enzima que inicia a mudança de isótipo e a hipermutação somática da imunoglobulina (Muramatsu et al., 2000), era também responsável pela reparação do ADN, permitindo assim às células B evitar a apoptose induzida por danos persistentes no ADN. Observámos (Wu et al., 2005) que a AID recruta a subunidade catalítica da ADN proteína quinase (DNA-PKcs) sugerindo que a AID coordena a reparação de quebras no ADN, contribuindo desta forma para a sobrevivência das células B.

Abstract

Our overall objective was to find mechanisms of B cell survival during antigen response. In the first part of this work, we revealed that immunoglobulin heavy chain expression is sufficient for B cell survival in the absence of light chain. We showed that the heavy chain alone can reach the cell surface as a part of an incomplete signal competent B cell receptor. These results are consistent with the hypothesis that the heavy chain alone can produce survival signals constitutively and independently of specific antigen recognition. We also showed an alternative hypothesis for B cell survival through the activation of an unfolded protein response, which is caused by heavy chain accumulation in the endoplasmatic reticulum in the absence of light chain.

In the second part of this work we looked for B cell survival mechanisms during activation and terminal differentiation. Specifically, how B cells survive DNA double strand breaks caused by class switch and somatic hypermutation of the immunoglobulin gene. Our results showed a novel role for the activation induced cytidine deaminase (AID), an enzyme responsible for class switch and introduction of somatic mutations in the immunoglobulin genes (Muramatsu et al., 2000). We showed that AID is involved in DNA repair by recruiting the catalytic subunit of the DNA protein kinase (DNA-PKcs) (Wu et al., 2005). These results suggest that AID coordinates double strand break repair and contributes to B cell survival during terminal differentiation

Keywords

IgL repressible mouse
Immunoglobulin Heavy Chain (HC)
B cell survival
B cell receptor (BCR) signaling
Unfolded protein response (UPR)
Activation induced cytidine deaminase (AID)
DNA protein kinase (DNA-PKcs)
Somatic Hypermutation
Class switch recombination (CSR)
DNA double strand break repair

Introduction

Adaptive immune responses of vertebrates have distinct properties from those of innate immunity universally exhibited by all multicellular organisms. Adaptive immunity is acquired, while innate immunity is inherited. Adaptive immunity evolves and forms an imprint of prior encounters, referred to as immunological memory, while innate immunity responses are fixed and repetitive, targeting highly conserved structures of known pathogens. Adaptive immunity mounts specific responses to an almost infinite number of varying structures displayed by infectious agents, toxins and other foreign molecules, while innate immunity responses are non-specific. The acquired specificity of adaptive immune responses depends on the availability of an enormous diversity of receptor molecules (B cell receptors and T cell receptors) that result from random assembly of precursor gene segments.

Immunological memory was, historically, the first feature of the adaptive immunity to be recognized (Panum, 1847). Although widely manipulated for the prevention of disease, the specific mechanisms necessary to develop and maintain immunological memory are still not completely understood today. One essential condition for the generation of immunological memory is that lymphocytes survive activation and persist quiescently even when antigen is no longer available for long periods of time. Upon re-exposure, the persisting lymphocytes must respond with enhanced kinetics and mount more effective responses in part owing to enhanced antigen binding capabilities compared to their naïve progenitors. This thesis concerns studies on the molecular mechanisms that contribute to the survival of B cells following activation.

In the early 20th century, Von Behring and Kitasato (Silverstein, 1999) discovered anti-toxins demonstrating that some properties of adaptive immunity could be transferred by sera. Ehrlich (Ehrlich and Morgenroth, 1957) called the humoral effectors, antibodies, and demonstrated that antibodies mount specific immune responses. Landsteiner and Chase (Landsteiner and

Chase, 1941) discovered in 1941 that immunity could be transferred by cells. Thus by mid-century the effectors of adaptive immunity antibodies and cells had been established as well as the fundamental properties of adaptive immunity: acquired, specificity and memory.

How B cells acquire and maintain long life is not known. Lam et al. (Lam et al., 1997), showed that conditional ablation of the VH exon in mature B cells, abolishing heavy chain expression led to the apoptosis of B cells. Kraus, M. et al. (Kraus et al., 2004) showed loss of mature B cells upon induced mutation of a signalling module of the BCR, not precluding BCR surface expression. The research suggested BCR signalling to be an absolute requirement for B cell survival.

Consistent with the idea that BCR signalling is needed for B cell survival, Kraus et al. (Kraus et al., 2004) showed that ablation of Ig signalling causes mature B cell death. However, B cells that lack a BCR exist under certain circumstances. For example, BCR-less B cells that express an Epstein Barr Virus receptor survive (Casola et al., 2004), and in camels (Muyldermans and Lauwereys, 1999) nurse shark (Greenberg et al., 1995) wobbegong shark and in ratfish (Rast et al., 1998) B cells lacking a conventional BCR live and secrete heavy-chain only antibodies. These studies suggested the possibility that at least under certain circumstances, expression of unpaired Ig heavy chain may sustain B cell survival.

The overall goal of this thesis was to discover elements contributing to the survival of B cells. We first examined the requirement of an intact BCR for the survival of B cells. To determine whether unpaired heavy-chain sustained B cell survival, we studied a mouse model engineered with a repressible Ig light chain gene (the Ig-L repressible mouse). We examined how repression of light chain in B cells that maintained heavy chain expression impacted on B cell survival. We found that survival of mature B cells did not require the expression of a complete BCR. B cells expressing Ig heavy chain alone were long-lived. Since Light-chain negative B cells expressed a signalling competent heavy chain on the surface of cells, we proposed that Ig

heavy chain signalling may be one mechanism contributing to B cell survival. While in most cells persisting unfolded protein responses target cells for destruction by phagocytosis, in B cells persisting unfolded protein responses are associated with terminal differentiation into plasma cells. Consistent with this concept, we found that repression of Ig Light chain expression enhanced differentiation of B cells into Ig heavy chain secreting cells. We concluded that unpaired heavy chains promote survival either by signalling from membrane complexes or by stimulating the unfolded protein response and differentiation of B cells. (Geraldes et al., 2007).

The generation of long-lived B cells is all the more intriguing considering that most differentiate after undergoing numerous DNA double strand breaks during class switch recombination and somatic hypermutation. Ordinarily, cells respond to DNA double-strand breaks by undergoing cell cycle arrest to allow time for repair (Nyberg et al., 2002) and respond to persistent DNA damage by inducing apoptosis, presumably as a protection against illegitimate recombination (Pfeiffer et al., 2000). How memory B cells that often express switched immunoglobulin isotypes and have mutations in the variable exons of the Ig heavy and light chains, survive multiple rounds of DNA breaks is not understood. In my thesis I examined whether activation-induced cytidine deaminase (AID) the enzyme that initiates somatic hypermutation (SHM) and class switch recombination (CSR) of Ig genes (Muramatsu et al., 2000) orchestrated DNA repair thus rescuing B cells from DNA damage induced apoptosis. Our studies (Wu et al., 2005) demonstrated that AID recruits DNA protein kinase, catalytic sub unit (DNA-PK_{cs}) suggesting that AID effectively coordinates repair with DNA breaks and in this way avoid apoptosis.

B cell development

B cell development, as generally viewed today, comprises two major phases marked by the properties of immunoglobulin (Ig) genes. In the first phase Ig heavy (H) and light (L) chain

genes are assembled from gene segments by V(D)J recombination to encode Ig heavy (H) and light (L) chains that constitute the B cell receptor (BCR). In the second, foreign antigen drives expansion of the B cells bearing a cognate BCR and they differentiate into either antibody-secreting plasma cells or into memory B cells.

Contrary to all other proteins with the exception of the T cell receptor, IgH and IgL chain genes are not encoded in the germline. In order to assemble a competent IgH or IgL gene B cells undergo DNA somatic recombination (called V(D)J recombination) which permits the random union of several V, D and J gene segments picked more or less randomly from a pool. The IgH chain V region is assembled first by joining a variable (V_H), a joining (J_H) and diversity (D_H) segment. The joining of D_H to a J_H segment occurs first, followed by the rearrangement of a V_H segment with to a DJ_H segment. Then the IgL chain rearranges by joining the variable (V_L) and joining (J_L) segments. V(D)J recombination H and L chain pairing potentially generates a BCR repertoire diversity of 10^{11} different receptors (Glanville et al., 2009; Jackson et al., 2013). V(D)J recombination results in the assembly of IgH and IgL variable exons that are brought to the proximity of the gene fragments encoding the Ig constant regions and to enhancer elements to drive effective transcription.

V(D)J recombination is initiated by a DNA cleavage step that is mediated by the recombination-activity genes 1 and 2 (RAG1 and RAG2) followed by DNA joining step mediated by non-homologous end joining pathway of DNA double strand repair (Helmink and Sleckman, 2012). The cleavage of DNA occurs adjacent to recombination sequences composed of heptamers and nonamers flanking a 12 or 23 non-conserved nucleotide spacer during the G1 stage of the cell cycle. DNA cleavage results in a pair of hairpin sealed coding ends and in a pair of blunt phosphorylated signal ends. The coding and signal ends are processed differently but remain in a complex until they can be joined. Joining of coding ends requires opening of the hairpins at a random position creating ends of different lengths which are filled in by addition of palindromic nucleotides (P). In addition the ends resulting from the opening of the hairpins are trimmed by exonucleases followed by addition of non-templated

nucleotides (N) (Helmink and Sleckman, 2012). Since processing of the coding ends includes variable addition and/or deletion of nucleotides the length of the junction will vary and only one third of the junctions will encode an in frame sequence (Helmink and Sleckman, 2012). Apart from generating a functional antigen receptor chain, coding joint formation also assures chromosomal integrity, necessary to preserve genomic stability. In contrast to coding joint formation, signal joints are joined precisely and in most cases form an extra-chromosomal circle that is lost upon cell division as it lacks replication ability. In the case of the Igκ locus, however, both coding and signal joints contribute to chromosomal integrity since VJ recombination causes inversion of the intervening sequences rather than deletion (Helmink and Sleckman, 2012).

The development of new B lymphocytes from their uncommitted precursors takes place in the bone marrow of adult mammals and is defined by the stepwise assembly of a competent BCR. Heavy (H)-chain genes are formed at the pro-B cell stage. Productive rearrangement of the VH segments leads to the expression of an IgH chain which is expressed on the surface in combination with a surrogate light chain to form the pre-B cell receptor. Only a minority (15%) of the proB cells transition successfully to the next stage. That is because only one out of every three heavy chain rearrangements places the VH gene segment in frame with the JH sequence, and because only one half of all the productive heavy chains pair with the surrogate light chain or mature light chains. Lack of heavy chain or pre-BCR expression causes arrest of B cells at the pro-B cell stage (Chen et al., 1993).

V(D)J rearrangement depends on VH gene accessibility and on the availability of Recombination activation genes 1 and 2 (RAG1 and RAG2). It is generally thought that transcription of germline VH sequences and chromatin remodeling render the VH and D segments accessible to the recombinase. This process requires the cytokine IL-7, and the transcription factors Pax5 and YY1. Transcription of the RAG genes depends on the function of Forkhead (FOX)p1 transcription factors(Kurosaki et al., 2010b).

The pre B cell receptor controls the clonal expansion of pre B cells. Pre-B cells undergo several divisions before rearranging the light (L)-chain genes. This rearrangement usually occurs first at the κ locus and then at the λ locus. Several attempts can be therefore made to rearrange a functional IgL chain and thus most pre-B cells survive to the immature B cell stage. Lack of L chain causes arrest at the pre-B cell stage of development (Spanopoulou et al., 1994; Young et al., 1994). Once a productive IgL chain is assembled and a complete BCR expressed on the surface, the pre-B cell becomes an immature B cell, which travels from the bone marrow through blood and lymph to the periphery, e.g., spleen, and lymph nodes where it further matures or dies.

We have discussed before that gene rearrangement is not an exact process so that it can give rise to a diverse repertoire of receptors. Thus, this process also gives rise to many non-productive rearrangements that need to be selected against in order to preserve the productive ones. Also, because in a diploid genome there are two alleles one in each immunoglobulin locus, a productive rearrangement must stop the process immediately and signal for the next developmental stage (allelic exclusion).

IgH allelic exclusion is the process by which successful rearrangement of one IgH allele inhibits further rearrangement of the other allele. IgH allelic exclusion is thought to occur after the transient surface expression of the newly rearranged functional IgH as part of the pre-B cell receptor (pre-BCR). The pre-BCR contains a functional IgH coupled with a surrogate light chain consisting of VpreB and λ5 (Keyna et al., 1995) (Kline et al., 1998) (ten Boekel et al., 1997). Non-productive IgH chains are unable to pair with surrogate light chains and are therefore retained in the endoplasmatic reticulum. The signals produced by the pre-BCR induce proliferation of the pre-B cell with a productive IgH rearrangement and provide a stop sign for further rearrangement (Hess et al., 2001) (Rolink et al., 2000). Although much

remains to be elucidated the mechanism requires Syk and Zap70 and PLCγ2(Kurosaki et al., 2010b).

Though a subject of controversy, pre-BCR-mediated proliferation of pre-B cells, in contrast to BCR-mediated proliferation of mature B cells, can occur independently of foreign antigens (Hess et al., 2001) (Rolink et al., 2000). Receptor self-aggregation is thought to be necessary for pre-BCR signaling. Thus Ohnishi and Melchers (Ohnishi and Melchers, 2003) proposed that the unique, non-Ig N-terminal tail of l5 and the C-terminus of V-PreB mediate homotypic interactions leading to pre-BCR clustering. The pre-BCR signaling renders the pre B cell independent of IL-7 for expansion and involves activation of Src family and Syk family kinases. The downstream events of pre-BCR signaling are yet to be clearly delineated (Kurosaki et al., 2010a). Pre-BCR mediated proliferation signals differ from those of mediated by the BCR of mature B cells in that the selection criteria is for a functional IgH chain irrespective of its specificity (Keyna et al., 1995) (Kline et al., 1998) (ten Boekel et al., 1997). Thus, a broader mechanism of selection is implied. About 12-15% of the pre-B cells arrange a functional IgH chain (ten Boekel et al., 1997). Interestingly, though SLC deficient pre-B cells expressing a functional IgH chain fail to proliferate efficiently, they can survive and differentiate (Hess et al., 2001) (Rolink et al., 2000).

After pre-B cell clonal expansion, pre-B cells arrest in G1 and become small pre-B cells. Since Pre-BCR signaling terminates the transcription of the genes encoding λ5 and V-preB, it is possible that the end of clonal expansion is causes by the depletion of complete pre-BCR receptors (Kurosaki et al., 2010b). Following cessation of proliferation, IgL loci become accessible, the rearrangement of the IgL chain follows and a complete functional IgM molecule is formed and expressed on the surface together with Igα and Igβ to form a functional BCR (Geier and Schlissel, 2006).

B cell selection and clonal deletion

The BCR determines whether a B cell lives or dies or whether it progresses to mount a productive immune response. How the BCR determines B cell survival is incompletely understood. A subject of controversy has been whether the BCR delivers a "survival signal" in the absence of a ligand (constitutive activation), or whether is continuously activated by endogenous ligands. The BCR also determines whether B cells become unresponsive (anergy), die (deletional tolerance) or whether they mount productive immunity to stimuli and differentiate into memory B cells or plasma cells. Thus, immature B cells that bind self-antigens are removed from the functional pool by clonal deletion (i.e., physical destruction) (Nemazee and Buerki, 1989) (Nemazee and Buerki, 1989) or undergo anergy (i.e., functional shutdown) (Berg et al., 1988), unless its L and/or H-chain genes are "edited" so that they no longer bind self-antigens (Nemazee, 2000).

In contrast, mature B cells that come in contact with foreign cognate antigen differentiate into antibody producing plasma cells or into-long-lived memory B cells. How B cells decipher whether a BCR signal should be interpreted, as a survival signal as opposed to a death signal, is not understood.

Survival of B cells following stimulation is determined by competition for antigen, T cell help and presumably by the availability of favorable niches that provide ideal growth conditions. Upon activation, B cells undergo frequent mutagenesis of the Ig variable heavy and light chain exons (Ig somatic hypermutation) (Wabl et al., 1985). This process changes the binding specificity of the BCR, may enhance affinity (the binding strength of each antigen binding site) of the BCR for antigen and creates intra-clonal diversity (Neuberger and Milstein, 1995). In addition, B cells undergo recombination and deletion of gene segments within the heavy chain constant region a process that mediates isotype class switch (von Schwedler et al., 1990). Somatic hypermutation introduces random point mutations in the rearranged V region

of Ig genes, allowing the generation of B cells with different antigen affinities within a clone (Cascalho et al., 1999). The diversity of B cells created by somatic hypermutation provides the small fraction of B cells with higher affinity for antigen with a survival advantage. The survival advantage may be contributed by enhancing antigen capture when antigen concentration is limiting, which in turn, may increase antigen presentation to cognate T cells enhancing T cell help (Schwickert et al., 2011) (Schwickert et al., 2011). In addition, B cells with receptors with higher affinity for antigen suppress the clonal expansion of low affinity B cells (Shih et al., 2002). Class switch recombination, exhanges the Ig heavy-chain Cμ region with one of the other heavy chain constant regions, resulting in the generation of antibodies of different isotypes and effector functions.

Somatic Hypermutation and class switch recombination are initiated by the activation induced cytidine deaminase (AID) encoded by the *AICDA* gene. AID is absolutely necessary to induce somatic hypermutation and/or isotype class switch because mice or human subjects that lack AID do not produced mutated antibodies, switched Ig isotypes and have deficient B cell memory (Muramatsu et al., 2000). Conversely transfection of the AID gene into fibroblasts is sufficient to induce mutation and switching events (Okazaki et al., 2002) indicating that AID works in cells other than B cells.

The function of AID as a cytidine deaminase was first proposed on the basis of its homology with the apolipoprotein B mRNA-editing catalytic polypeptide 1 (Kim et al., 2002). Exactly how AID introduces point mutations or executes class switch recombination is not yet completely understood, but it is generally thought that cytidine deamination of DNA (or RNA) somehow generates double-strand breaks in Ig DNA (Durandy, 2003) (Papavasiliou and Schatz, 2000) (Petersen et al., 2001) (Rogosch et al., 2012) (Celeste et al., 2002).

Ordinarily, cells respond to DNA double-strand breaks by undergoing cell cycle arrest to allow time for repair (Nyberg et al., 2002) and respond to persistent damage by inducing

apoptosis, likely as a protection against illegitimate recombination (Pfeiffer et al., 2000). However, B cells undergoing Ig class switch do not die, presumably because they efficiently repair DNA double-strand breaks. Although RAD54, RAD52, and RAD51 repair proteins are needed for AID-induced Ig gene conversion in chicken cell lines (Bezzubova et al., 1997) (Sale et al., 2001), whether AID directly recruits repair factors to the locales of cytidine deamination is not known. In our work, we identified one possible mechanism contributing to AID-mediated recruitment of DNA repair factors (Wu et al., 2005).

B cell memory

Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously. Enhanced responses might reflect the persistence of antigen-specific B cells bearing receptors that bind antigen with high affinity that remain quiescent following antigen clearance (memory B cells) and/or persistence of antigen specific plasma cells (long-lived plasma cells).

It is generally accepted that a primary encounter with antigen stimulates specific B cells (the cells that produce antibodies) not only to differentiate into plasma cells, but also to give rise to populations of long-lived memory cells (Gray, 1988) and long lived plasma cells (Arce et al., 2002). Memory B cells differ from naïve B cells in their lifespan and in the properties if the immunoglobulin (Ig) genes which are often class switched and have somatic mutations in the V exons (Meffre et al., 2001). Memory B cells contribute to the rapidity of the secondary responses as the number of memory antigen-specific B cells is greater than the number of antigen specific naïve B cells (Ahmed and Gray, 1996), and because responses from memory B cells occur faster than from naïve cells in part owing to an increased affinity B cell receptor for the antigen and to specific properties of the IgG BCR enhancing sensitivity and rapid activation (Martin and Goodnow, 2002) (Engels et al., 2009; Liu et al., 2010). Long-lived plasma cells are thought to maintain antigen-specific antibodies in the blood long after the

antigen has cleared. Because plasma cells don't express surface Ig they do not contribute to the properties of recall responses. Recent evidence suggests that plasma cells negatively regulate the follicular T helper program inhibiting further activation of antigen-specific B cells (Pelletier et al., 2010).

The fundamental requirement for generation of B cell memory is that antigen specific B cells (memory B cells or long lived plasma cells) persist after stimulation. However the number of antigen-specific B cells persisting after stimulation probably decreases with time indicating that memory B cells form a heterogeneous population with diverse lifespans.

In part, the uncertainties on the frequency of memory B cells and on how long they live are a consequence of a lack of consensus on a phenotype. Bona fide memory cells were defined as cells that carry somatic mutations in the V exons of the Ig genes (Dietrich et al., 1989) (Ahmed and Gray, 1996) (Bikah et al., 2000). This is because hypermutation of the V exons occurs upon B cell activation and clonal expansion, and because mutated B cells that bind antigen better have a survival advantage. However, since mutation *per se* is not required a better definition of memory B cell reflects their history and unique properties. Thus, Shlomchik and Weisel (Shlomchik and Weisel, 2012) recently defined memory B cell as the "member of a clone that has responded to antigen by proliferation, remaining in the animal in a resting state and at expanded frequency long after the initial stimulus".

In contrast to naïve B cells, memory B cells survival and activation is independent of B-lymphocyte stimulator (BlyS or BAFF) (Good et al., 2009) (Scholz et al., 2008), and independent of T cell help (Hebeis et al., 2004) (Klinman and Doughty, 1973). Alternative markers have been sought but controversy remains on whether there is a common phenotype to all B cell memory cells (Balin and Cascalho, 2010) (McHeyzer-Williams et al., 2000). Some (Shlomchik and Weisel, 2012) defined subsets of memory B cells based on the expression of several surface markers including CD12/23, CD73, CD80 and PD-L2 (CD283) but their functional significance remains unclear.

A matter of debate is whether the maintenance of memory B cells requires continuous stimulation by antigen (Gray, 1988). Maruyama and colleagues (Maruyama et al., 2000) addressed this question with an elegant genetic switch. Based on their findings the authors concluded that maintenance of B cell memory occurs independently of antigen. However since the "new" Ig expressed by the memory B cells could in principle, still bind the immunizing antigen, the issue of whether B cell memory depends on antigen persistence or simply expression of Ig is not solved.

A longstanding question in B cell memory is how cells in a clone decide to become either long-lived memory B cells or plasma cells. Three models have been proposed: (i) memory cells arise from a lineage different from the cells that constitute the primary response (Linton et al., 1989); (ii) memory cells arise from the same lineage of plasma cells and plasma cells are generated at low frequency as the clone grows (linear differentiation pathway) (Ahmed and Gray, 1996); (iii) as clones grow, the potential for memory cell development decreases (decreasing potential hypothesis) (Ahmed and Gray, 1996). While the jury is still out on which model best describes reality, Pape et al. (Pape et al., 2011) showed that while both antigen specific IgM and switched Ig memory B cells were generated, these cells differed in their responses to repeat stimulation and in their longevity. Thus the more numerous IgM+ cells were longer lived than the swig+ cells and responded to re-stimulation by forming germinal centers. In contrast, swig+ B cells provided early memory responses by quickly originating antibody-secreting cells but very few germinal centers. The results by Pape et al. suggested that the properties of memory B cells evolve with clonal expansion and with time after antigen encounter and support the model first proposed by Ahmed and Gray (Ahmed and Gray, 1996) suggesting that the potential for memory B cell development decreases with clonal expansion.

In addition to memory B cells, long-lived plasma cells constitute the survivors of primary responses. Plasma cells are terminally differentiated B cells that lost Ig surface expression and for that reason are un-responsive to antigen (Manz et al., 1998). Thus, their sole function appears to be maintaining a low level of specific Ig in circulation to confer protection against pathogens or toxins while secondary responses get started (Slifka and Ahmed, 1998) (Manz et al., 1998) (McHeyzer-Williams and Ahmed, 1999). Long-lived plasma cells may last a life time (Crotty et al., 2003) (Slifka and Ahmed, 1998) and Ahuja et al. (Ahuja et al., 2008) showed that long lived plasma cells are maintained independently of memory B cells because extended depletion of memory B cells did not decrease the number of plasma cells in the bone marrow or spleen, or the steady state specific antibody levels. Instead, survival of long-lived plasma cells might depend on environmental factors in the bone marrow defining an optimal niche. Consistent with that idea Sze et al. (Moulin et al., 2000) determined that the spleen has the capacity to sustain between 20 and 100 plasma cells/mm^2 beyond which cells are lost. When this number is exceeded, there is a loss of excess cells. Although the origin of long-lived plasma cells has been linked to the germinal center, research from several laboratories suggests that these cells also arise in response to polysaccharide, T-independent antigens which typically do not stimulate germinal center formation (MacLennan et al., 2003) (Obukhanych and Nussenzweig, 2006) (Garcia de Vinuesa et al., 1999) (Taillardet et al., 2009).

Memory B cells prolonged longevity compared to naive B cells may depend on the properties of membrane-bound IgG (mIgG) compared to those afforded by membrane bound IgM (mIgM), more frequently expressed by naive B cells. Differences in signalling mIgG and by membrane bound IgM mIgM can be explained in part owing to differences in their respective sequences. IgM and IgG BCRs have different extracellular, transmembrane (TM) and cytoplasmic Fc (fraction crystallisable) domains (Kurosaki et al., 2010a). The Fc region of membrane-bound IgG, is shorter than that of membrane IgM and the extracellular antigen binding sites of IgG are linked to the Fc region by flexible hinge region (Arnold et al., 2007). Membrane bound IgM, encodes a more rigid Cμ2 domain containing a conserved Asparagine (N46)-linked glycosylation site that is absent in the flexible hinge region of membrane IgG

(Arnold et al., 2007). The N46 glycosylation of membrane bound μHC and the arginine rich tail of λ5 light chain in the pre-BCR are necessary for spontaneous signalling to drive B cell development(Ubelhart et al., 2010). N46 glycosylation is not necessary for BCR signalling in mature B cells.

While both IgM- and IgG-BCRs require Igα/Igβ heterodimers to engage the B-cell signalling apparatus through immunoreceptor tyrosine activation motifs (ITAMs) in the Igα/Igβ cytoplasmic domains (Reth, 1992),(Reth, 1992 the extracellular domains of Igα/Igβ heterodimer associates better with IgM than with IgG {Radaev, 2010 #4209). The functional consequences of preferential association of Igα/Igβ to IgM are not known.

Both mIgM and mIgD have three amino-acid cytoplasmic tails. In contrast, all mIgG subtypes have long cytoplasmic tails of 28 amino acids, which are highly conserved across species,(Xu et al., 2014) and references there in. Consistent with the idea that IgG cytoplasmic tail is necessary to contribute some of the enhance responsiveness of memory B cells are studies by Kaisho et al. (Kaisho et al., 1997) and by Martin et al. (Martin and Goodnow, 2002). Kaisho et al. (Kaisho et al., 1997)showed that tailless mIgG1 transgenic mice had significantly decreased specific antibody responses compared to the transgenic mice expressing the wild type mIgG1. Martin et al. (Martin and Goodnow, 2002) showed that a transgenic mouse expressing the ectodomain of IgM and TM and cytoplasmic tail of IgG1 generated enhanced memory responses compared to the mice expressing the IgM wild type transgene. In addition, Waisman et al. (Waisman et al., 2007) showed that IgG1 can exert a unique signalling function that can partially replace that of the Igα/Igβ heterodimer to promote B cell survival. These results indicate that the isotype of membrane bound Ig determines B cell responsiveness and survival and may explain some of the properties of B cell memory responses.

Antigen-induced B cell signalling pathways

Antigen binding to the BCR induces signalling which assure entry in the cell cycle, cell survival during proliferation, metabolic synthesis to support growth, antigen presentation to ensure continuous stimulation and orchestrates B cell fate decisions (Cambier et al., 1994; Dal Porto et al., 2004; DeFranco, 1997; Kurosaki et al., 2010b).

Multivalent antigen binding induces BCR oligomerization and results in signal transduction that depends on the non-covalently BCR associated Igα/Igβ heterodimer (Kurosaki et al., 2010b). After binding of multivalent antigen, the cytoplasmic domains of the BCR components also cluster. Then the Igα and Igβ cytoplasmic domains are separated by a considerable distance, transitioning from a closed to an open conformation. The open conformation is dependent on ITAM phosphorylation, most likely by the action of Lyn, one of the Src family PTKs. BCR engagement activates three different families of protein tyrosine kinases, Src, Syk and Tec. Activation of Src kinases causes tyrosine phosphorylation of the immuno-tyrosine activation motifs (ITAM) in the cytoplasmic domains of Igα/Igβ which in turn bind the src homology-2 (SH2) domain of a second kinase, Syk resulting in auto-phosphorylation.

BCR clustering occurs in the context of the immunological synapse, which is characterized by the central accumulation of BCR and antigen, termed the central supramolecular activation cluster (c-SMAC), surrounded by a ring of adhesion molecules (peripheral SMAC, or p-SMAC). Intracellular signaling molecules such as Syk, phospholipase Cγ2 (PLCγ2), and Vav1 are co-localized with the initial BCR-containing microclusters (Depoil et al., 2008). CD19 is transiently recruited into the BCR clusters and is important for amplifying BCR signaling (44). These observations suggested that the micro-signalosomes (probably including CD19, Syk, PLCγ2, PI3K, and Vav) are a critical site for the initiation of BCR signaling. At a later time point (15 min after stimulation), the micro-signalosomes dissociate from the BCR clusters that reside in the c- SMACs.

BCR- signalling results in the triggering of at least four effector pathways: PLCγ2/calcium/NFAT pathway; PI3K pathway; IKK/NF-κB pathway and; ERK pathway

PLCγ2/calcium/NFAT pathway

Syk phosphorylation generates docking domains for the recruitment and activation of several other downstream molecules such as the Bruton tyrosine kinase (Btk) and phospholipase Cγ2 (PLCγ2). PLCγ2 hydrolyzes phosphatydyl-inisitol 4,5 biphosphate (PIP2) to generate two important effectors: inositol 1,4,5 triphosphate (IP3) and dyacyl glycerol (DAG). IP3 triggers Ca++ mobilization from the ER which when depleted trigger Ca++ import from the exterior. Elevation of Ca++ in the cytosol is sensed by Calmodulin which when bound to Ca++ activates several molecules including Calcineurin phosphatase and Nuclear-factor of activated T cells (NFAT). NFAT requires continuous elevated Ca++ to remain in the nucleus where it directs gene transcription including interferon regulatory factor 4 (IRF4) a critical transcription factor to direct plasma cell differentiation (Klein et al., 2006; Sciammas et al., 2006).

PI3K pathway

Following BCR ligation the cytoplasmic tail of CD19 can be phosphorylated by lyn creating binding sites for the SH2 domains of the p85 subunit of PI3K, Vav and Lyn (Brooks et al., 2000; Fujimoto et al., 2000; Tuveson et al., 1993). Activation of PI3K requires tyrosine phosphorylation of the B cell adaptor for PI3K (BCAP). CD19 and PI3Kp110δ are necessary for the development of peritoneal B1 cells, spleen marginal zone B cells and CD19 is required for the development of spleen CD1dhiCD5+ B cells (Akagi et al., 1999; Fruman et al., 1999; Janas et al., 2008). CD1dhiCD5+ B cells are thought to produce IL-10 and therefore contribute to immune-regulation (Yanaba et al., 2008). Thus the CD19 component of the B cell signalossome also engages regulatory mechanisms .

Activated PI3K converts PIP2 into PIP3 which in turn serves as an anchoring domain to activate proteins with a plekstrin homology domain such as Akt. Akt activates the mammalian target of rapamycin (mTOR) to enhance protein synthesis and cellular metabolism, and inactivates FoxO that is necessary to maintain quiescence in resting B cells. In this way Akt helps to drive proliferation of resting B cells (Xu et al., 2014).

IKK/NF-κB pathway

Stimulation of the BCR induces PLCγ2 activation which in turn produces DAG. DAG recruits protein kinase C family members that activate CARMA1, Bcl10 and MALT1 (Thome, 2004) causing activation of the IKK complex (consisting of IKKα, IKKβ, and IKKγ or NEMO), thereby phosphorylating IκB and inducing its degradation (Hacker and Karin, 2006). Degradation of IKB allows NF-κB transcription factors that are retained in the cytoplasm by binding to IκB, to translocate to the nucleus. The NF- κB pathway mediates pleotropic functions that control proliferation, survival and differentiation of activated B cells. The NF- κB pathway is subject to tight control at many different levels. For example, the association of CARMA1 with Bcl10 and MALT1 depends on its phosphorylation on Ser668 by PKCβ (Shinohara et al., 2005). Other Ser/Thr phosphorylation sites in CARMA1 and Bcl10 also contribute to the control of NF- κB activation. In another example, activated IKKβ phosphorylates CARMA1 on Ser578 which stabilizes the CARMA1, Bcl10 and MALT1 complex (Shinohara et al., 2007). Phosphorylation of Bcl10, on the other hand, causes its degradation and inactivation of the CARMA1, Bcl10 and MALT1 complex {Lobry, 2007 #4311}.

ERK pathway

DAG generated by PLCγ2 in response to BCR activation facilitates mobilization of both guanine nucleotide exchange factors and PKCβ to the membrane. PKCβ activates RasGRP3 by Thr133 phosphorylation. Upon activation, RasGRP3 stimulates the mitogen activated

proteins kinases (MAPK) resulting in ErK translocation to the nucleus and transcription (Kurosaki et al., 2010b).

The repressible Ig-lambda LC mouse

To elucidate the elements of B cell memory, we engineered a novel experimental system in mice, the repressible Ig-lambda LC mouse. In the repressible Ig-lambda LC mouse, all B cells contain an antigen receptor of a defined specificity that can be reversibly repressed. In addition, its T cells express the transgenic DO.11 TCR specific for an ovalbumin peptide. Because it was engineered in a RAG-negative background both B and T cells are monoclonal. The lambda light chain expression will be repressed with doxycycline (a tetracycline derivative). The control system consists of two elements: a transactivator and a target gene. The transactivator acts upon a minimal promoter in the absence of tetracycline (or its derivative doxycycline) in the target gene. The minimal promoter (P min) and elements of the bacterial tet operon (tetO) are cloned to a structural gene of choice that is to be regulated. In a cell line (or mouse) constitutively expressing HSV VP16 fusion protein, which functions as a transactivator (tTA), the gene of choice will be expressed so long as there is no doxycycline. Doxycycline binds to the tTA, and thus its presence represses gene expression. Several transactivator constructs have been developed. We have chosen a transactivator transgenic mouse in which expression of the tTA is under the control of the mouse mammary tumor virus long terminal repeat (MMTV-tTA). The nature of the reversibly repressible system that controls the expression of the light chain makes it particularly suited for the study of the immune response. It allows not only an on-off switching, but also a more subtle modulation of expression levels. The expression of tetracycline inducible transgenes has been shown to be regulated over a range of up to three orders of magnitude in mice expressing MMTV- tTA (Hennighausen et al., 1995) (Redfern et al., 1999) and in mice expressing the tTA under the control of a MHC II promoter (Witherden et al., 2000). The synthesis of the Ig heavy chain will be directed by the knock-in VH17.2.25DJH4 exon targeted to its correct location on the

chromosome to allow developmentally and quantitatively correct B cell development, as well as isotype switching and somatic hypermutation.

The tetracycline-dependent transactivator system

Regulation of expression of the lambda gene was engineered using the tetracycline-transactivator system. The tetracycline-dependent transactivator system includes an inducible promoter that works in a variety of human and murine cells, and whose activity can be regulated over five orders of magnitude (Gossen and Bujard, 1992) (Furth et al., 1994) (Kistner et al., 1996) (St-Onge et al., 1996). The control system consists of two elements: a transactivator and a target gene. The transactivator acts upon a minimal promoter in the absence of tetracycline (or its derivative doxycycline) in the target gene. The minimal promoter (P min) and elements of the bacterial tet operon (tetO) are cloned to a structural gene of choice that is to be regulated. In a cell line (or mouse) constitutively expressing HSV VP16 fusion protein, which functions as a transactivator (tTA), the gene of choice will be expressed so long as there is no doxycycline. Doxycycline binds to the tTA, and thus its presence represses gene expression.

Several transactivator constructs have been developed. A transactivator transgenic mouse in which expression of the tTA is under the control of the mouse mammary tumour virus long terminal repeat (MMTV-tTA) was chosen. Transcriptional activation using the tetracycline regulatable system has been shown to be completely abrogated in the presence of doxycycline and to be regulated over a range of up to three orders of magnitude in mice expressing the MMTV-tTA transactivator (Hennighausen et al., 1995) (Redfern et al., 1999) and in mice expressing the tTA under the control of a MHC II promoter (Witherden et al., 2000). The MMTV-LTR targets the expression of tTA to lymphocytes and to epithelial cells of the secretory organs (Hennighausen et al., 1995) (Redfern et al., 1999). In these models, B cell memory will be generated by immunization with a hapten-carrier conjugate (NP-ovalbumin).

The NP System

The hapten (4-hydroxy-3-nitrophenyl) acetyl (abbreviated NP), which induces an immune response restricted in idiotype, has been extensively studied (Imanishi and Makela, 1974) (Karjalainen, 1980) (White-Scharf and Imanishi-Kari, 1981) (White-Scharf and Imanishi-Kari, 1982). The VH gene segments encoding these idiotypes, including VH17.2.25 (Loh et al., 1983), were cloned. Antibodies produced during primary responses to NP are almost exclusively of the λ type; thus, the 17.2.25 heavy chain produces antibodies specific for NP when it combines with a λ light chain. There are only three Vλ segments in the mouse, Vλ1, Vλ2 and Vλx (Azuma et al., 1981) (Blomberg et al., 1981) (Miller et al., 1981) (Selsing et al., 1982) (Elliott et al., 1982) (Reilly et al., 1984) (Dildrop et al., 1987) (Sanchez and Cazenave, 1987). I found that both Vλ1 and Vλ2 bind to NP when associated with VH17.2.25 (Cascalho et al., 1996). Whether or not Vλx does so as well has not been tested. In addition, the VH17.2.25/λ antibody does also recognize NIP (4-hydroxy-2-iodo-3-nitrophenyl) acetyl, and pNP (4-nitrophenyl) acetyl but with different affinities.

The T cell receptor transgenic system

The DO.11.10 TCR-transgenic mouse line was developed in Dr. Loh's laboratory in 1990, and since then has been widely studied (Murphy et al., 1990). Transgenic T cells express the TCR from a T cell hybridoma, DO11.10, that recognizes the chicken ovalbumin peptide (OVA323-339) in the context of H-2^d and H-2^b (Murphy et al., 1990) (Liu et al., 1996). DO.11.10 TCR T cells mature into CD4-positive T cells in mice expressing H-2^d. However, in mice expressing H-2^b, an important fraction of the peripheral T cells expresses neither CD4 nor CD8 (Liu et al., 1996). The transgenic TCR expressed in this system can be detected by the clonotypic monoclonal antibody KJ1-26 (Marrack et al., 1983) and by a monoclonal antibody that binds Vβ8, F23.1. In this proposal, DO 11.10 is abbreviated DO.11.

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IG HEAVY CHAIN PROMOTES MATURE B CELL SURVIVAL IN THE ABSENCE OF LIGHT CHAIN[1]

Pedro Geraldes[*], Michelle Rebrovich[*], Kai Herrmann[¶], Jamie Wong[‖], Hans-Martin Jäck[¶], Matthias Wabl[‖] and Marilia Cascalho[*,†,‡,§,2]

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Transplantation Biology Program[*] and the Departments of Immunology[†], Surgery[‡] and Pediatrics[§], Mayo Clinic College of Medicine, Rochester, Minnesota 55905, USA; Division of Molecular Immunology[¶], Nikolaus-Fiebiger Center, Friedrich-Alexander-University Erlangen-Nürnberg, Glückstrasse 6, 91054 Erlangen, Germany;

Department of Microbiology and Immunology[‖], University of California, San Francisco 94143-0670

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Abstract

Survival of mature B cells is thought to depend on the B cell receptor signaling (BCR) because ablation of either Heavy chain (HC) expression or BCR signaling causes B cells to rapidly disappear. Whether a complete BCR is required for survival of mature B cells is not known. To address this question we generated a mouse in which we can repress the expression of a transgenic Ig Light chain (IgL) by doxycycline (IgL repressible mouse). Repression of IgL abrogated BCR expression. Surprisingly, however, IgL-negative B cells survived longer than 14 weeks, expressed signal-competent HC on the cell's surface and active unfolded protein response (UPR) factors. Like post-germinal center B cells, IgL-negative B cells were small lymphocytes, not dividing and expressed Bcl-6. Our results indicate that expression of unpaired HC as it may occur as a consequence of antigen ligation, somatic hypermutation or receptor editing, facilitates the survival of cells either by inducing receptor signaling, or by inducing UPR and/or the expression of survival genes such as Bcl-6.

Introduction

The development and survival of mature B cells is thought to require stable expression of surface immunoglobulin (Ig) (Lam et al., 1997) and expression of functional Igαβ dimer (Kraus et al., 2004). Lam et al. (Lam et al., 1997) and Kraus et al. (Kraus et al., 2004) showed that repression of heavy chain (HC) and consequently of surface Ig expression causes death of mature B cells and inferred that survival depends on B cell receptor (BCR) signaling through the Igαβ dimer, much as the T cell receptor is required for T cell survival (Anderson et al., 1999). Whether instead the HC itself might promote survival independently of the BCR was not formally considered. We sought to test this question.

Expression of membrane-bound HC drives early B cell differentiation even in the absence of a complete surrogate light (SL) chain and conventional light (L) chains. This idea is supported by the studies of Schuh et al. (Schuh et al., 2003) who found that a transgenic μHC reaches the cell surface in the absence of the SL chain component λ5 and conventional L chains induce IL-7-dependent cell growth and promote *in vivo* differentiation of pro-B cells. Similarly, Galler et al. (Galler et al., 2004) showed that μHC signals terminate the expression of terminal deoxynucleotidyl transferase and downregulate the expression of the recombination activation genes 1 and 2 (RAG1 and RAG2) in the absence of SL and L chains.

B cells of camels, sharks, and ratfish produce HCs that cannot pair with LCs (Conrath et al., 2003). In these species (Conrath et al., 2003) and when expressed in mice (Zou et al., 2005), unpaired HCs appear to drive B cell development and contribute to HC-only antibodies which make up to 75% of the serum Ig (Conrath et al., 2003). Synthesis of HC-only antibodies may depend on some HC unique features including V_H FR2 domain adaptations and lack of a C_H1 domain. These features antagonize binding to LCs and possibly to the chaperone immunoglobulin HC binding protein (BiP) that retains unpaired HCs in the endoplasmatic reticulum (ER) (Conrath et al., 2003) thus enabling trafficking from the ER to the cell surface

in the absence of LC. Synthesis of HC–only antibodies suggests that unpaired HCs may sustain B cell development and mature B cell survival.

In light of these properties of HC we questioned whether murine HC expressed without LC might sustain mature B cell survival. To test this concept, we generated a novel experimental system in mice: the IgL-repressible mouse. In the IgL-repressible mouse, expression of LC and of surface BCR can be abrogated by feeding the mice doxycycline. Expression of HC remains unaffected in these mice. From the phenotype of the IgL-repressible mouse, we here report that, contrary to expectations, mature B cells survive repression of LC and that continued expression of HC alone drives long-term survival of B cells. We also report that HC is expressed on the surface of cells and can associate with Igαβ dimers to yield a functional complex that promotes survival. In contrast to studies by Corcos et al. (Corcos et al., 2001) showing that truncated heavy chains that lack the V_H exon are expressed unpaired on the surface of B cells, B cells of the IgL-repressible mouse express the full-length protein. Although truncated heavy chains do not sustain survival of B cells (Corcos et al., 2001) our studies indicate that expression of full-length HC does. These findings may explain how some cells of B lineage (e.g., plasma cells or neoplastic B cells) survive with little or no surface Ig.

Materials and Methods

Mice

The IgL-repressible mouse has monoclonal B and T cell compartments. Because the mice are on a recombination activation gene 1 negative background (RAG1-/-), no endogenous B or T cell antigen receptors are produced. Instead, T cells express the transgenic DO.11.10 βαTCR (Liu et al., 1996; Loh et al., 1983; Murphy et al., 1990), while B cell immunoglobulin is encoded by a combination of knock-in μHC gene (VH17.2.25) (Cascalho et al., 1996) and a λLC transgene (Young et al., 1994). The transgenic T cell receptor is specific for an ovalbumin peptide (OVA aa323-aa339) and the combined transgenic knock-in B cell receptor is specific for the hapten 4-hydroxy-3-nitrophenyl acetyl (abbreviated NP) and its derivatives (Cascalho et al., 1996). Expression of λLC is regulated by the availability of doxycycline. In the absence of doxycycline a transactivator binds the minimal promoter and drives λLC expression (Figure 1A).

The LC gene was engineered using the tetracycline-inhibitable transactivator system (Gossen and Bujard, 1992). This system consists of a transactivator and a target λLC transgene. The transactivator or tTA (HSV VP16 fusion protein) is under the control of the mouse mammary tumor virus long terminal repeat promoter (MMTV-tTA) and is constitutively expressed. The MMTV-LTR targets the expression of tTA to lymphocytes and to epithelial cells of the secretory organs (Hennighausen et al., 1995; Redfern et al., 1999).

The transactivator (tTA) in the absence of tetracycline (or its derivate doxycycline) acts upon the λLC transgene minimal promoter (Pmin) promoting transcription. Doxycycline binds the tTA, preventing it from binding Pmin (Hennighausen et al., 1995; Redfern et al., 1999) and effectively repressing λLC expression.

Tetracycline-responsive λ1 transgene

The repressible lambda construct puts the λLC gene under the control of a tetracycline and transactivator-responsive promoter (TetO). The λ gene was obtained by Eco R1 digest of the C2 plasmid (a kind gift of Dr. Fay Young (Young et al., 1994)). This fragment contains the lambda endogenous promoter and the VλJλ rearrangement linked to Cλ in a genomic configuration. The promoter region was subsequently excised by further digestion with Sex A1, which cuts 15 base pairs upstream of the start codon ATG. The 5.8 Kb fragment was then blunted and cloned at the PvuII site of the pBI-EGFP plasmid (Clontech, cat#6154-1). The final plasmid puts the λ gene under the control of a TetO regulatory element linked to the hCMV minimal promoter. Because the λ gene lacks an intronic enhancer and there is no 3' λ enhancer in this construct, transcription depends on the binding of a transactivator to the TetO element. EGFP expression which in the pBI-EGFP plasmid is under the control of a TetO regulatory element was lost upon breeding the founder mice.

Generation of the IgL repressible mouse by breeding

The λ-repressible founders were mated to the MMTV-tTA mouse and to mice of the following genotype: RAG1-/-, V_H T/ V_H T, DO11 TCR, H-$2^{d/d}$. The repressible Ig mice, obtained from the previous crossings, have the following phenotype: RAG1-/-, V_H T/ J_H +, λ ind, tTA, DO11-TCR, H-$2^{b/b}$. The studies here discussed were performed with mice derived from two independent founders.

Animal care and doxycycline treatment

All mice were between 1 to 3 months of age and kept in a specific pathogen-free facility at the Mayo Clinic. All animal experiments were carried out in accordance with protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee. IgL repressible mice were fed doxycycline diet (grain based) 200mg/Kg (S3888) (Bio-Serv, Frenchtown, NJ).

Genotyping

DNA was extracted from mouse tails according to standard protocols (Cascalho et al., 1996). Genotyping was done by PCR amplification of tail DNA with thermoprime plus DNA polymerase (ABgene, Rochester, NY) and 12 pmol of each primer for 40 cycles using an Icycler (Biorad, Hercules, CA) thermocycler. H chain knock-in (V_H17.2.25 DJ_H4) Forward-5'AAGTTCAGCTGCAGCAGTCTGG 3'; reverse-5'GGGACAAATATCCAAGATTAGTC 3', 450 base pairs, Tm 51°C; Lambda 1 LC Forward-5' GCCTTTCTACACTGCAGTGGGTATGCAACAAT 3'; Reverse- 5' AGCCACTYACCTAGGACAGTSASYTTGGTTCC 3', 500 base pairs, Tm 60°C; TTA Forward-5' AGAGAATGCATTATATGCACTCAGCG 3'; Reverse- 5' AGACCCGTAATTGTTTTTCGTACGCG 3', 280 base pairs, Tm 55°C; TCR Forward-5' CAGGAGGGATCCAGTGCCAGC 3'; Reverse-5'TGGCTCTACAGTGAGTTTGGT, 300 base pairs, Tm 52°C; I-A^b Forward-5' CATAGCCCCAAATGTCTGACCTCTGGAGAG 3'; Reverse- 5' AGTCTTCCCAGCCTTCACACTCAGAGGTAC 3', 200 base pairs, Tm 60°C; and I-A^d Forward-5' CATAGCCCCAAATGTCTGACCTCTGGAGAG 3', Reverse- 5' CATGGGCATAGAAAGGGCAGTCTTTGAACT 3', 200 base pairs, Tm 60°C.

Cell lines and culture conditions

Ag8.H cells were grown in complete RPMI (RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 5% FCS, 1 mM sodium pyruvate and 2 mM L-glutamine. Ag8.H-Igα transfectants were selected in complete RPMI supplemented with 1 mg/ml G418 (PAA, Pasching, Germany), whereas Ag8.H-Igα expressing µHC was cultured in complete RPMI with 1 mg/ml G418, 1.25 µg/ml mycophenolic acid, 250 µg/ml xanthine, 100 µM hypoxanthine and 16 µM thymidine. All cell lines were maintained at 37°C and 5% CO_2 in a humidified incubator.

The Ag8.H-Igα cell line was generated to analyze the cell surface transport of B cell receptor (BCR) complexes by transfection of Ag8.H, a subclone of the murine immunoglobulin-

negative plasmayctoma cell line Ag8.653 (Kearney et al., 1979). Murine Igα, required for surface transport of BCR complexes (Hombach et al., 1988), was amplified by PCR from cDNA of the murine lymphoma B-cell line CH27 with an appropriate forward (TTGGATCCACGATGCCAGGGGGTCTAGA, containing a *Bam*HI restriction site) and backward primer (TTGAATTCCAGTCATGGCTTTTCCAGCT, containing an *Eco*RI restriction site). The Igα fragment was cloned into the *Bam*HI and *Eco*RI site of the mammalian expression vector pEF1/myc-His (Invitrogen, Karlsruhe, Germany) and transfected by electroporation (250 V, 960 µF; (Keyna et al., 1995)). Subsequently, pµ.gpt encoding an IgL-pairing and functional µHC with the same VH17.2.25DJH4 variable exon of the HC expressed in the repressible Ig mouse (Jack et al., 1992) were transfected in Ag8.H-Igα by electroporation. Stable cell clones were established by limiting dilution and analysed by flow cytometry.

Flow Cytometry

Organ cell suspensions were prepared by pushing the organ through 0.70 µm mesh (spleens and lymph nodes) or by passing the tissue repeatedly though a 27G needle (bone marrow). White blood cells were isolated using Ficoll-Plaque (GE Healthcare) gradient and cells counted with a Coulter counter (Beckman Coulter, Miami, FL). Surface staining of splenocytes were done as described (Cascalho et al., 1996); for intra-cytoplasmic staining splenocytes were fixed in PBS supplemented with 2% paraformaldehyde at 4°C for 1 hour, permeabilized in 1 ml of 0.2% Tween 20 in PBS and in 1 ml 0.1% NaN_3 with 2% fetal calf serum. For membrane staining of cultured cells 5 x 10^5 cells were incubated for 30 min on ice in PBS supplemented with 2% FCS and 0.1% NaN_3 with the appropriate amount of antibodies. For cytoplasmic staining of cultured cells, 5 x 10^5 cells were fixed in 4% paraformaldehyde in PBS at RT for 10 min, permeabilized with 0.1% Tween20 at 37°C for 15 min and stained at RT for 15 min with the appropriate amount of antibodies. Data analyses were performed using a FACSCalibur (BD Biosciences, San Jose, CA) and CellQuest™ software (v4.0.2).

Antibodies

Antibodies used were biotin-labelled, goat IgG directed against the murine IgM (H+L), purchased from Southern Biotech (Birmingham, AL), from Becton Dickinson (Franklin Lakes, NJ) APC (Allophycocyanin)-labeled anti-mouse CD19 (1D3), biotin labeled anti-mouse Ig, λ, λ2 & λ3 LC (R26-46), R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD23 (FcεRII) (B3B4) monoclonal antibody, Fluorescein Isothiocyanate (FITC)-conjugated rat anti-mouse CD21/CD35 (CR2/CR1, CD21a/CD21b) (7G6) monoclonal antibody, FITC-conjugated rat anti-mouse CD24 (Heat Stable Antigen) (M1/69) monoclonal antibody, biotin-conjugated mouse anti-mouse IgD^a (Igh-5a, Igh-5.4) (AMS 9.1) biotin-conjugated mouse anti-mouse IgD^b (Igh-5b) (217-170) for B6 mice, and FITC-conjugated rat anti-mouse CD45R/B220 (RA3-6B2) monoclonal antibody. Biotin-labeled antibodies were detected with Streptavidin-PE-Cy5 (BD Biosciences Pharmingen, Franklin Lakes, NJ). Ki67 was detected with rat anti-mouse Ki67 (TEC-3, DAKO) antibody in tissue sections.

Unconjugated affinity purified goat antibodies against mouse IgM (H+L) (Birmingham, AL) were labelled with the Cy5 labelling kit from Amersham Biosciences (Freiburg, Germany). The monoclonal mouse IgG1,κ antibody 24C2.5 against the intracellular tail of mouse Igα was previously described (Mielenz et al., 2003).

Tunel assay

Done with an apoptosis detection kit, ApopTag Red in Situ, according to the manufacturer's instructions (Chemicon, Int)

Ca2+ influx

Ca2+ influx studies were performed by incubating splenocytes with 5μM INDO-1 AM (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature and then labelled with anti-CD19 and anti-B220 antibodies for 30 min at 4°C. Cells were kept at 37°C for 2 min before

adding unlabeled the stimulus: polyclonal unlabeled goat anti-mouse IgM (H+L) (200μg/ml) (Southern Biotech, Birmingham, AL), polyclonal unlabeled goat anti- mouse IgM $(Fab')_2$ (25μg/ml) (Southern Biotech, Birmingham, AL), polyclonal unlabeled goat anti-mouse lambda (λ chain specific) (200μg/ml), polyclonal unlabeled total goat anti-mouse IgG were purchased from Southern Biotech, Birmingham, AL. Ionomycin was obtained from Calbiochem, MERK KGaK, Darmstadt, Germany. Data collected and analyzed on a cytometer (LSR II, Becton and Dickinson, Franklin Lakes, NJ) with Flow Jo software. The results are shown in indo1-violet/indo1-blue ratio.

RT-PCR and Real Time PCR

RNA was extracted using Qiagen RNeasy Kit (Valencia, CA, cat# 74104) according to the manufacturer's instructions. The RNA yield was measured with the nanodrop ND-3300 Fluorospectrometer (Wilmington, DE). Reverse transcription was performed using Invitrogen ThermoScript RT-PCR System (Carlsbad, CA, cat# 11146-016) also according to the manufacturer's instructions. Real time PCR was performed in the Roche Light cycler using QuantiTect SYBR Green PCR Kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. The cDNA was amplified using the following primers: Pax-5 F CTACAGGCTCCGTGACGCAG and Pax-5 R TCTCGGCCTGTGACAATAGG (annealing 65°C 439bp), VpreB F GTCTGAATTCCTCCAGAGCCTAAGATCCC and VpreB R CAGGTCTAGAGCCATGGCCTGGACGTCTG (annealing 60°C 400bp), Lambda5 F GGGTCTAGTGGATGGTGTCC and Lambda5 R CAAAACTGGGGCTTAGATGG (annealing 60°C 205bp), VH F GGGATATCCACACCAAACATC and VH R CATACACAGAGCAACTGGACA (annealing 50°C 1785bp), myc for CAGCTCTGGAGTGAGAGGGGCTTT and myc rev GTAAGTTCCAGTGAGAAGTGTCTG (annealing 59°C 150bp), Ire1 F AGAAGCTACCTGTTGGCCGTTGTA and Ire1R CATCCTGGAAGAACTGGAGCTCCT (annealing 59°C 150bp), mChopRT3 TGCAGGGTCACATGCTTGGC and mChopRT2 GCCTGACCAGGGAGGTGGAG (annealing 54°C 150bp), Edem1 F ATCCGAGTTCCAGAAGGCAGT and Edem1 RV GCTTCCCAGAACCCTTATCGT (annealing 53°C 150bp), mBiPRT1 GATTCCAAGGAACACTGTGGTA and mBiPRT3

CCAGTCAGATCAAATGTACCC (annealing 52°C 150bp), λ T9 F CCAGGCTGTTGTGACTCAGGA and λ tg CDR2 R GGAGCTCGGTTGTTGGTACCA (annealing 54°C 150bp), AID3 ATCTCAGACTGGGACCTGGAC and AID5 CCTTGCGGTCTTCACAGAAGT (annealing 53°C 174bp), Xbp1u F AGCACTCAGACTATGTGCACCTCT and Xbp1s R GGACATTTGAAAAACATGACAGGG (annealing 58°C 163bp), Xbp1s F TGCTGAGTCCGCAGCAGGTGCA and Xbp1s R GGACATTTGAAAAACATGACAGGG (annealing 58°C 150bp), BLIMP1 F TGACTTTGTGGACAGAGGCCGAGT and BLIMP1 R CTGTTGTTGGCAGCATACTTGAAA (annealing 58°C 150bp), Bcl6 for TGCAGGAAGTTCATCAAGGCCAGT and Bcl6 rev TTCTCAGTGGCATATTGTTCTCCA (annealing 58°C 150bp) and finally β-actin F CCTAAGGCCAACCGTGAAAAG and β -actin R TCTTCATGGTGCTAGGAGCCA (annealing 54°C 600bp). The Real time primers where Lambda 1 CDR2 GGAGCTCGGTTGTTGGTACCA and Lambda TG Fw CCAGGCTGTTGTGACTCAGGA (173bp) and LCM β -actin 1 AGCTGGCCGGGACCTAACTGACTA and LCM β -actin 2 AGCCGTGGCCATCTCTTGCTCGAA (151BP).

Immunohistochemistry

Done essentially as described in João et al. (João et al., 2004). The primary antibodies used were: Unlabeled goat anti-mouse lambda LC and FITC-conjugated goat $F(ab')_2$ anti-mouse IgM (H+L) were purchased from Southern Biotech (Birmingham, AL), AP was purchased from Cappel (Irvine, CA), rat anti-mouse CD19 (1D3) purchased from BD Biosciences-Pharmingen (Franklin Lakes, NJ) and rat anti-mouse CD180 (RP105) (RP/14) purchased from Serotec (Raleigh, NC). The secondary/tertiary antibodies used were: Rhodamine-conjugated Donkey $F(ab')_2$ anti-rat IgG (H+L) from Jackson ImmunoResearch (West Grove, PA), FITC-conjugated rabbit $F(ab')_2$ anti-goat IgG from ICN/Cappel (Irvine, CA) and FITC-conjugated goat $F(ab')_2$ anti-rabbit IgG from ICN/Cappel (Irvine, CA). Slides were examined on a fluorescence microscope (Leica DMRD, Bannockburn, IL). Digital images were obtained utilizing a high-resolution CCD digital camera (SPOT II, Diagnostic Instruments, Sterling Heights, MI) mounted to the microscope and SPOT II software.

Immunoprecipitation and Western Blot analysis of splenocytes

Spleens were harvested and cell suspensions prepared by pushing homogenates through a 70 µm nylon mesh. Red blood cells where depleted using Ficoll-Paque (GE Healthcare). B cells were isolated using the MACS column (Miltenyi Biotec, Auburn, CA) and the B cell isolation kit (Miltenyi Biotec, Auburn, CA) yielding 3 to $10x10^6$ cells per preparation. Protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL) as per the manufacturer's instructions. When surface biotinylation was performed, $10x10^7$ cells in 250µl PBS were incubated with 4 mg of Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 30 minutes at RT. Cells were washed with PBS supplemented with 100 mM glycine to quench and remove excess biotin. Cells were lysed in BEACH buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5mM EGTA, 5 mM EDTA, 15 mM MgCl, 60 mM β-glycerolphosphate, 1 mM DTT, 0.1 mM Na-vanadate, 0.1 mMNaF, 15 mM p-nitrophenyl phosphate, 1% Triton X-100 and 1 proteinase inhibitor tablet from Roche, Basel, Switzerland) for 30-45 min on ice. Cell lysate corresponding to 3 to$10x10^6$ cells was centrifuged at 14000 rpm at 4°C for 20 min and the supernatant was collected for analysis.

For immunoprecipitation, 35 µl of beads were coated with 1-5µg of the appropriate antibody for 2h at 4°C with agitation. Coated beads were incubated with the cell lysate obtained from 3 to$10x10^6$ cells, for 2h at 4°C with agitation and after washing, extracted with 35 µl of 2X sample buffer at 100°C for 5 min. Samples were analyzed on a 10%SDS PAGE and blotted onto an Immobilon–P 0.45 µm PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat milk in PBST for 45 min at room temperature. Blotted proteins were revealed with a primary antibody, incubated overnight at 4°C followed by washing and a secondary antibody incubated for 45 min at RT. After washing with PBS, blots were developed using lumiglo reagent purchased from Cell signaling (Danvers, MA).

The primary antibodies were HRP or biotin conjugated, affinity purified goat anti-mouse IgM, human absorbed (Southern Biotech, Birmingham, AL), rabbit IgG anti-mouse lambda LC (ICN0029) and goat anti-mouse IgG (γ chain specific) (Southern Biotech, Birmingham, AL). The secondary reagents were HRP-conjugated Protein A (Amersham Biosciences, Pittsburgh, PA), HRP-conjugated anti-mouse Ig (H&L) and HRP-conjugated anti-goat Ig (Cell Signaling, Danvers, MA). Beads for immunoprecipitation were Streptavidin immobilized on 4% beaded agarose (Sigma, St. Louis, MO), Protein A agarose (Sigma, St. Louis, MO) and Protein G (Pharmacia).

Western Blot analysis of cultured cells

1 x 10^6 cells were washed in 2 x PBS and lysed on ice for 30 min with NET buffer (150 mM NaCl, 1 mM sodium vanadate, 50 mM NaF, 0.5% Nonidet P-40, 1mM PMSF, 5 mM EDTA, 25 mM Tris-HCl (pH 7.4)). Solubilized cells were centrifuged (10.000 x g for 10 min at 4°C) and supernatants analyzed by SDS-Page according to Laemmli et al. (Laemmli, 1970). Broad Range Marker (Bio-Rad, Munich, Germany) was used as molecular weight standard. Separated proteins were O/N transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in 25 mM Tris, 192 mM glycine and 20% methanol at 4°C. Membranes were blocked with 5% non-fat milk in TBST pH 7.5 (25 mM Tris-HCl, 150 mM NaCl and 0.1% Tween20) for 1h. The blots were incubated with HRP-conjugated goat anti-mouse IgM (H+L) (Southern Biotech, Birmingham, AL) diluted 1:6.000, for 1h. Blots were washed for 4 x 10 min in TBST and developed with the ECL method.

ELISA

Total splenocytes where lysed using Beach Buffer. Serial dilutions of lysates were incubated overnight at 4°C in ninety-six well flat bottom microtiter plates (Nunc-Immuno 96 Micro well – Maxisorp, Bioscience, San Diego, CA) coated with 2 μg/ml of unlabeled goat anti-mouse lambda (Southern Biotech, Birmingham, AL) and blocked with PBS supplemented with 0.1% gelatin and 0.1% Tween20. Lambda LC was revealed with HRP-conjugated goat anti-mouse

lambda (Southern Biotech, Birmingham, AL) and developed with ABTS. Plates were read at 405 nm at 5, 10 and 20 min on a microplater reader (Power Wave X, Bio-Tek Instruments, Winooksi, VT) and analyzed using the software KC4 – Kineticalc for Windows.

Results

The IgL repressible mouse

The IgL repressible mouse is bred onto a recombination activation gene-1 negative background (RAG1-/-). B cells in this mouse express a monoclonal BCR consisting of a constitutively expressed knock-in μHC (VH17.2.25) (Cascalho et al., 1996) and of a DOX-repressible λ1LC transgene (Young et al., 1994). Expression of λ1LC transgene is driven by the binding of a tetracycline-controllable transcription factor (tTA) to the minimal tet promoter (Pmin) (Gossen and Bujard, 1992; Hennighausen et al., 1995; Redfern et al., 1999). The activity of the tTA can be abolished by the addition of tetracycline or its derivative doxycycline (Figure 1A). Since the tTA gene is under the control of the mouse mammary tumor virus long terminal repeat promoter (MMTV-tTA), the expression of tTA is constitutive and targeted to lymphocytes and to epithelial cells of the secretory organs (Hennighausen et al., 1995; Redfern et al., 1999). The transgenic BCR is specific for the hapten (4-hydroxy-3-nitrophenyl) acetyl (abbreviated NP) and its derivatives (Cascalho et al., 1996). T cells in the IgL-repressible mouse are also monoclonal and express the DO.11 10 αβ TCR (Murphy et al., 1990).

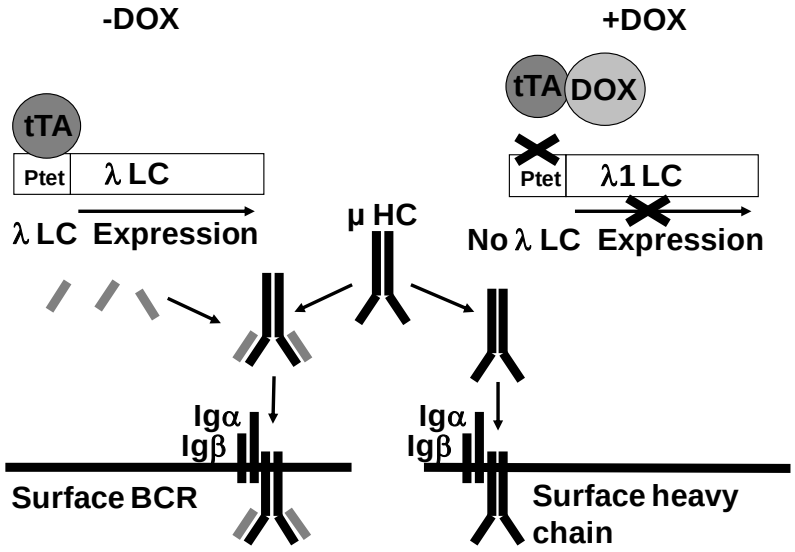

FIGURE 1A: Proposed model of λLC repression in IgL-repressible mice. Briefly: in the absence of doxycycline, the constitutively expressed transactivator (tTA) binds the tet promoter and drives expression of λLC; when doxycycline is present it binds the transactivator (tTA) and prevents it from binding to the tet promoter effectively abrogating λLC expression. The μHC continues to be expressed following doxycycline treatment and reaches the surface with Igα/β.

Doxycycline treatment represses LC production

We first determined whether administration of DOX to the IgL-repressible mouse inhibits expression of λLC. λLC protein could not be detected by Western Blot analysis of splenocytes isolated from mice that were fed doxycycline for four weeks (Figure 1B, lane 3). ELISA of homogenates (including cytoplasmic and membrane bound protein) obtained from purified B cells confirmed the absence λLC in DOX-treated IgL-repressible mice. ELISA limit of detection of λLC was 2ng/ml. when all B cells expressed λLC (in the QM mouse), we measured 64.6 ng/ml or 23 ng/million B cells. We detected only 5.5 ng λLC /ml in control C57BL/6 B cells corresponding to 8.5% of the QM values, which is in linewith the fact that only 5-10% of B cells in B6 mice express λLC.

Repressible-Ig mouse B cells can only express λLC. In the absence of DOX, we detected 19.0 ng λLC/ml or 1.5 ng λLC/million B cells, indicating that B cells in the repressible-Ig mouse produce 15-fold less λLC than QM B cells. The assay could not detect λLC above background levels in as many as 0.5 X 10^6 B cells obtained from a repressible-Ig mouse following DOX treatment. This result indicated that DOX-treated repressible-Ig B cells produced at least 32-fold less λLC as aQM B cells and had at least 3-fold less λLCthan non DOX-treated repressible Ig B cells.

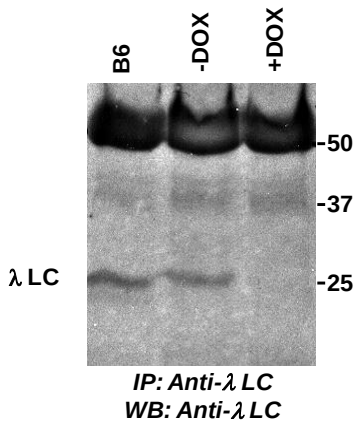

FIGURE 1B: Western Blot analysis of λLC expression. Splenocyte cell lysates were obtained from B6 or from pooled IgL-repressible mice not fed or fed doxycycline, as indicated. Lysates were immunoprecipitated with goat anti-mouse λLC antibody and revealed with the same antibody on Western Blots. Equal amounts of protein were loaded in each well (0.5mg).

To determine the extent of transcription repression of the λLC gene in mice fed doxycycline, we performed RT-PCR with primers that were specific for cDNA. We failed to detect LC mRNA (Figure 1C).

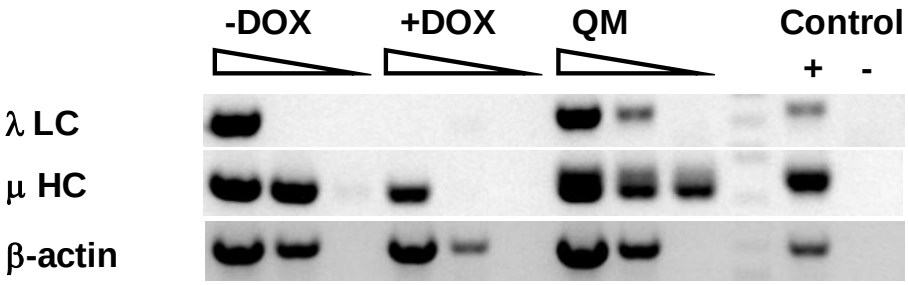

FIGURE 1C: RT-PCR analysis of λ1 LC and μHC mRNA obtained from total splenocytes of IgL-repressible mice fed or not fed doxycycline or from QM mice, as indicated. Figure shows the absence of detectable λLC mRNA in doxycycline-treated mice. μHC and β-actin were used to control the quantity and integrity of cDNA. cDNA obtained from 1 μg of RNA was PCR amplified from serial dilutions (1:1, 1:5 and 1:25), for 35 cycles.

To determine if repression of λLC protein expression effectively abrogated LC function as part of the BCR, we compared changes in the level of intracellular free Ca^{2+} in B cells stimulated

with anti-λLC antibody. While B cells from IgL-repressible mice not treated with doxycycline responded to anti-λLC antibody by quickly increasing the intracellular Ca^{2+} (Figure 1D, blue line), B cells from mice treated with doxycycline did not (Figure 1D, red line). These results indicate that doxycycline treatment generated B cells functionally lacking LC, which we will refer to as "LC–negative B cells."

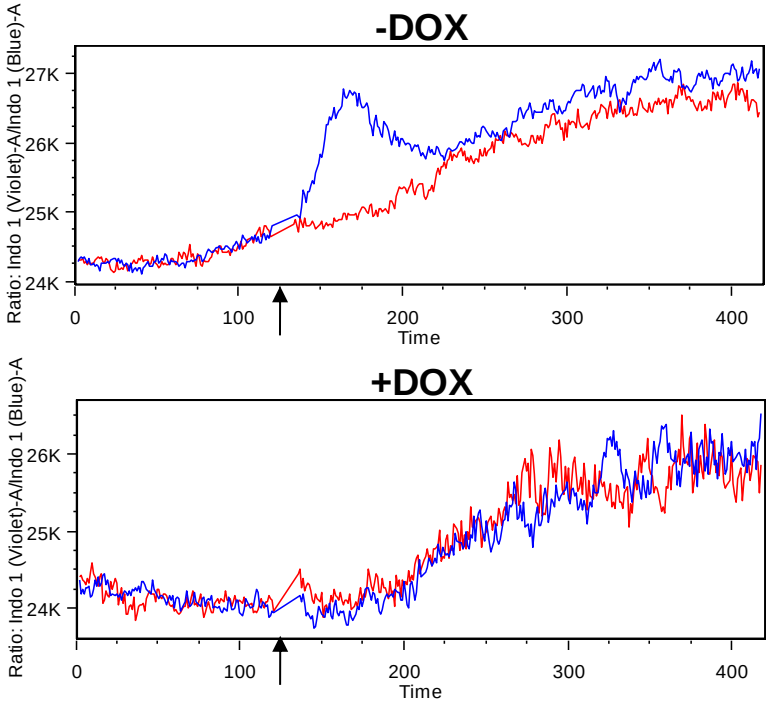

FIGURE 1D: Flow cytometry analysis of Ca^{2+} influx in splenocytes obtained from IgL repressible mice treated (below) or not treated (above) with doxycycline. Histograms represent ratio of fluorescence intensities of Indo 1AM bound to Ca2+ / free Indo 1AM over time (seconds). Arrows denote the time the stimulus was added, goat anti-mouse λ LC antibody (blue) or goat IgG control (red). LC-repressed splenocytes failed to generate a rapid Ca^{2+} influx peak following LC cross-linking (bottom diagram).

B cells survive and continue to express membrane bound λHC upon LC repression

Expression of surface BCR is thought to require the correct assembly of heavy and light chains. Hence, we questioned whether repression of LC affects surface μHC expression on splenic B cells isolated from doxycycline-treated mice. As expected from the analysis of λLC expression by Western Blot analysis, we detected surface λLC on all CD19-positive

splenocytes from mice not fed doxycycline (Figure 2A, first row), but not from mice fed doxycycline. Only about 5% of B6 splenocytes expressed λLC (Figure 2A, first row, diagram on right). However, despite the complete repression of LC expression in mice fed doxycycline, we detected μHC in the cytoplasm as well as on the surface of B cells (Figure 2A, second and third row). Surface μHC was reduced by 10-fold in B cells that lack LC (Figure 2A, second row), but cytoplasmic μHC was not.

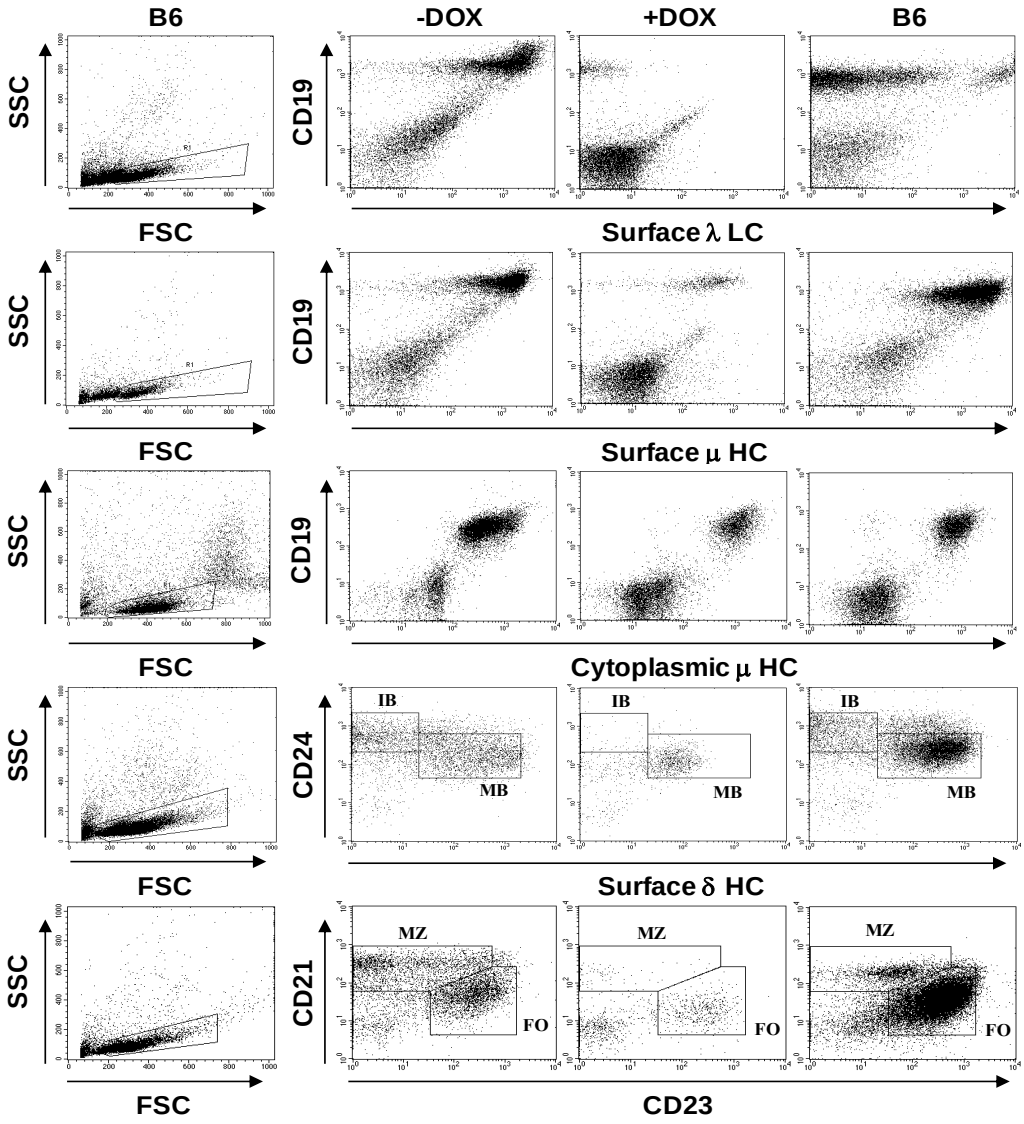

FIGURE 2A: Flow cytometric analysis of splenocytes from IgL repressible mice fed or not fed doxycycline and B6 control, as indicated. The left column represents the lymphocyte gate on the light scatter plot used in the analysis. The three columns to the right represent the fluorescence intensity plots of lymphocyte gated events stained with labeled antibodies, as indicated. Surface and cytoplasmic μHC were detected with a goat anti-mouse

IgM(H+L), all other antibodies were rat anti-mouse monoclonal. Mature (MB) and immature (IB) B cells were identified in fluorescence intensity plots shown in row 4 obtained from lymphocyte and CD19-positive gated events. Marginal zone (MZ) and follicular (FO) B cells (fifth row) were identified in fluorescence intensity plots shown in row 5 obtained from lymphocyte and B220-positive gated events. The figure shows that λLC-positive, surface CD19-positive B cells disappear in mice treated with doxycycline but CD19-positive, λ LC-negative cells survive (upper row) expressing both surface (second row) and cytoplasmic (third row) μHC. Immature and marginal zone B cells are absent in doxycycline treated IgL repressible mice. Only 5% (circa) of CD19-positive B cells in B6 mice are λ LC positive.

The presence of cells with markers characteristic for B cells (CD180, CD19 and μHC) could be verified in the absence of LC in histological sections of spleen obtained from IgL-repressible mice fed doxycycline (Figure 2B). These data show that LC-negative cells express CD180, CD19 and μHC indicating that B cells survived repression of LC. To determine which subsets of B cells survived repression of LC we distinguished mature ($CD19^{+}/CD24^{lo}/IgD^{hi}$) immature ($CD19^{+}/CD24^{hi}IgD^{lo}$), marginal zone ($B220^{+}/CD21^{hi}/CD23^{lo}$) or follicular B cells ($B220^{+}/CD21^{lo}/CD23^{hi}$) by flow cytometry analysis of splenocytes obtained from IgL-repressible mice fed or not doxycycline. Figure 2A fourth row shows that immature B cells (IB) and marginal zone B cells (MZ) disappeared following LC repression. The surviving B cells included follicular B cells, the numbers of which decreased from 1.4 million to 0.3 million, 4 weeks after LC repression.

Pre-B cells that express HC do so in conjunction with surrogate LC (Melchers et al., 1993). The surrogate LC is composed of λ5 and V pre-B proteins, and these together with HC form the pre-B cell receptor that reaches the cell surface (Iglesias et al., 1993; Melchers et al., 1993). Because the pre-B cell receptor is thought to sustain survival of pre-B cells (Rajewsky, 1996), we asked whether B cells surviving repression of LC express a pre-BCR. Figure 2C shows that mRNA for the surrogate LC components Vpre-B and λ5 was absent in peripheral LC-positive as well as in LC-negative B cells. These results were corroborated by flow cytometry using antibodies specific for pre-BCR components (not shown) and indicate that surface LC-negative B cells were not pre-B cells. Since surviving B cells expressed CD19 and

the Pax-5 transcription factor (Figure 2C), and plasma cells do not (Delogu et al., 2006), our results also indicate that LC-negative B cells were not terminally differentiated.

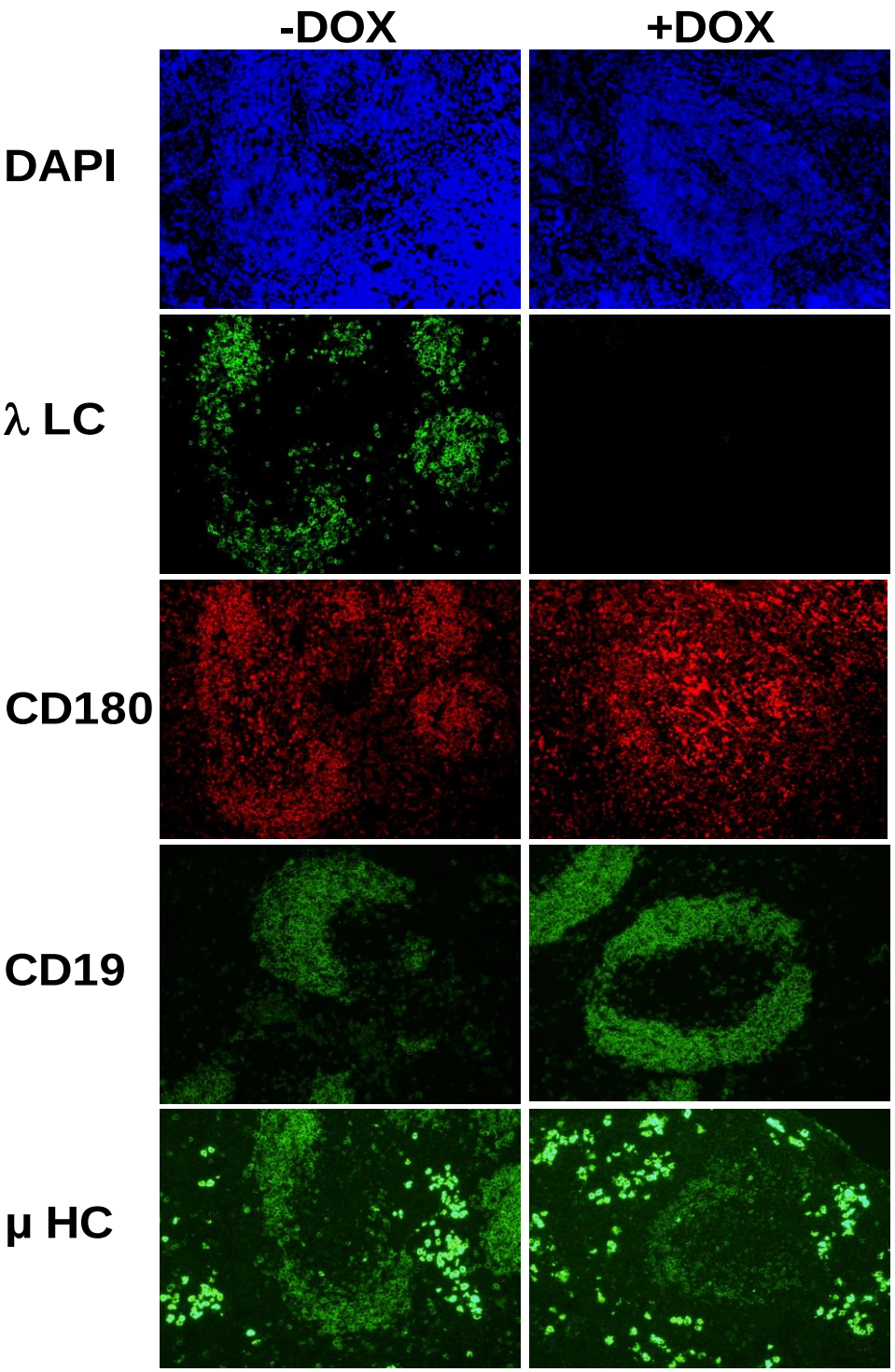

FIGURE 2B: Frozen sections obtained from spleen of IgL repressible mice fed (right) or not fed (left) doxycycline. Sections were co-stained with anti-λ (FITC-conjugated) (second row) and anti-CD180 antibodies (Rhodamine-conjugated) (third row). Sections shown in the fourth row were stained with an anti-CD19 antibody (FITC-conjugated) and in the fifth row, with an anti-IgM(H+L) antibody (FITC-conjugated), as indicated. All sections were also stained with DAPI to identify nuclei as shown in the first row. Figure shows that λ LC expression is abrogated by doxycycline and that surviving B cells that λ LC repression express CD180, CD19 and μHC.

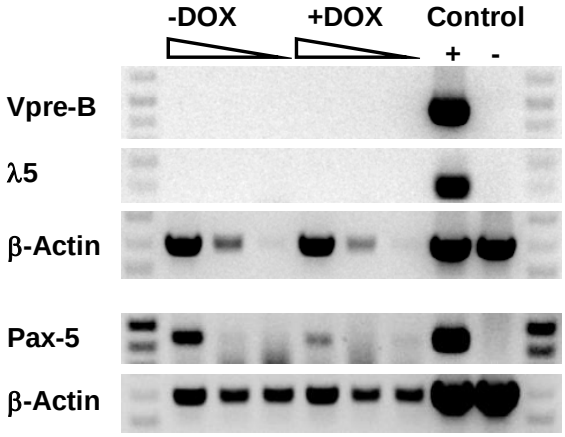

FIGURE 2C: RT-PCR analysis of Pax-5, Vpre-B and λ5 mRNA obtained from IgL repressible mice fed or not fed doxycycline, as indicated. Pax-5 expression marks B cell lineage, whereas the absence of both Vpre-B and λ5 expression rules out pre-B cells. PCR was done with 1:1, 1:5 and 1:25 cDNA dilutions.

To confirm that the transgenic VH17.2.25 chain reaches the cell surface in the absence of IgL chains, we transfected SL and L chain-negative Ag8.653 first with a gene encoding Igα and then with a vector encoding the μH chain (VH17.2.25-μHC) identical to the one expressed in the IgL-repressible mouse (Figure 2D). Figure 2E shows that the VH17.2.25-μHC is transported to the surface of cells in the absence of LC.

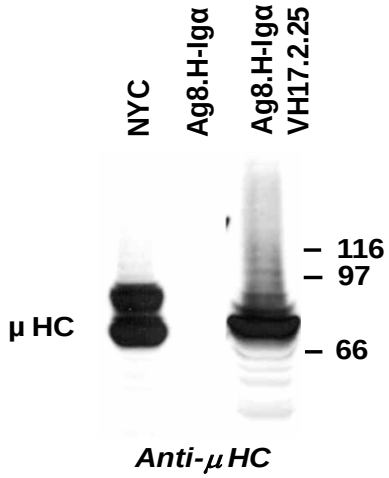

FIGURE 2D: Analysis of μHC expression in Ag8.H clones by Western Blot. Ag8.H cell lines where transfected with a construct expressing Igα and another construct expressing the μHC VH17.2.25, the HC in the repressible IgL mouse that pairs with Igα. The mature B cell line NYC served as positive control.

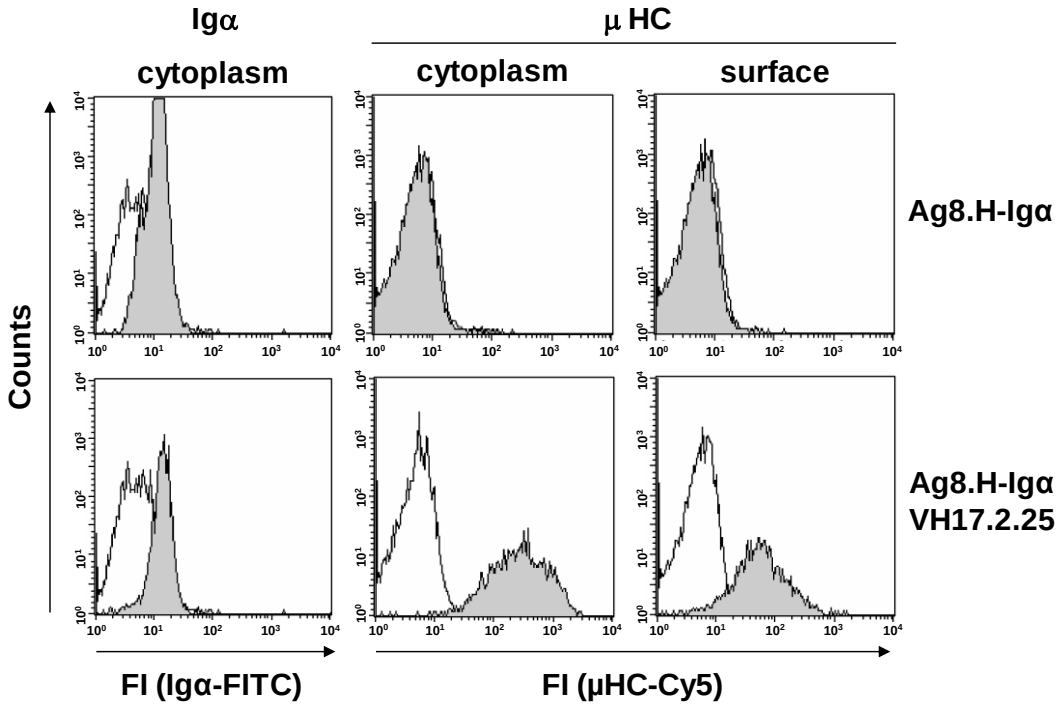

FIGURE 2E: Flow cytometry analysis of μHC VH17.2.25 surface and cytoplasmic expression in Ag8.H clones. Intracellular and membrane expression of Igα, and μHC in Ag8.H clones. Untransfected Ag8.H-Igα served as

negative staining control (non-filled line). Left column: Stable Ag8.H transfectants were cytoplasmic stained with 24C2.5 antibodies against the intracellular tail of mouse Igα, FITC-conjugated antibodies against mouse Fcγ served as secondary antibodies (grey-filled line). Right column: Stable Ag8.H transfectants producing VH17.2.25 μHC were cytoplasmic or membrane stained with Cy5-conjugated antibodies against mouse μHC (grey histograms). Figure shows that μHC VH17.2.25 is expressed on the surface. Results shown are representative of three independent experiments.

Surface LC-negative B cells are long lived

The survival of pre-B cells and the survival of mature B cells is thought to depend on expression of a surface receptor (Lam et al., 1997; Reichlin et al., 2001). Lam and colleagues (Lam et al., 1997) and Kraus and colleagues found that repression of HC abolishes the expression of a complete BCR and causes death of B cells. Whether B cells die because of the absence of surface BCR or to the absence of HC on its own was not determined. In contrast, repression of LC in the IgL-repressible mouse abolishes the assembly of a complete BCR but does not affect surface μHC expression. To determine how long LC-negative B cells expressing neither BCR nor pre-BCR survive, we enumerated B cells in the spleen and in the peripheral blood of mice at different times following LC repression. The number of B cells in the spleen decreased 5-6 fold (from 2.4 to 0.4 million) four weeks after LC repression but it remained constant thereafter. The decrease in the number of B cells in the spleen was not observed in the peripheral blood. Figure 3A shows that the number of CD19-positive B cells in the blood remained constant for up to 14 weeks after the start of the DOX treatment. Long-lived B cells expressed *bcl-6, blimp-1*, activation induced cytidine deaminase *(aid)* and did not express *c-myc* mRNAs consistent with a non-cycling, post-germinal center phenotype (Figure 3B). Expression of *aid* and *blimp-1* suggests the possibility that these factors are needed to establish or maintain survival of B cells in a non-terminally differentiated state.

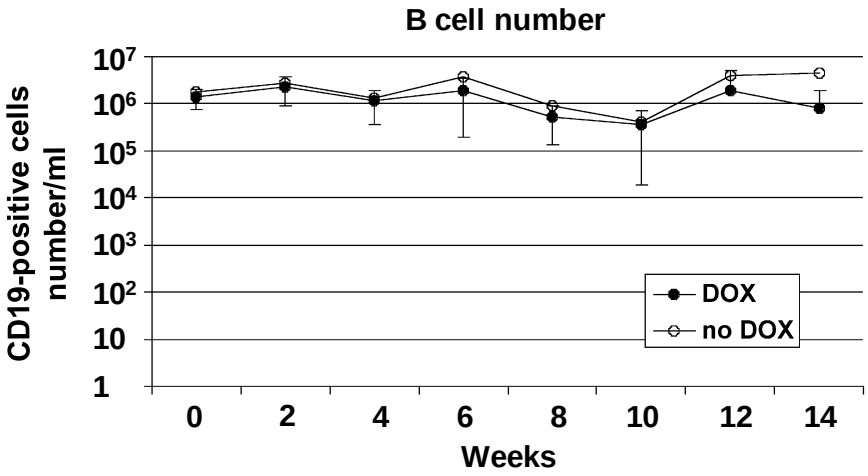

FIGURE 3A: CD19-positive B cells survive up to 14 weeks following the start of doxycycline treatment and repression of λ LC as their number remains constant in the peripheral blood of IgL repressible mice. Cells were enumerated by coulter counting and flow cytometry analysis of peripheral blood lymphocytes with rat monoclonal antibody anti-mouse CD19 APC-labeled. Figure shows the average number of CD19-positive B cells obtained from 6 different mice treated with doxycycline and one untreated mouse analyzed at each time point.

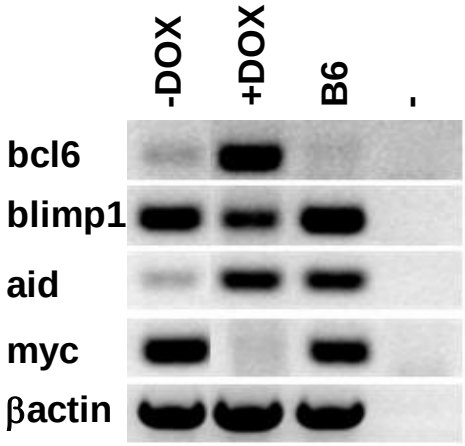

FIGURE 3B: RT-PCR analysis of mRNA obtained from CD19-positive B cells isolated by MACS purification from pooled spleens of 6 repressible IgL mice fed or not fed doxycycline for 2 weeks. Surviving B cells express *bcl-6*, *blimp-1*, activation induced cytidine deaminase *(aid)* and do not express *c-myc* mRNAs consistent with a non-cycling, post-germinal center phenotype. To control for DNA contamination of RNA samples, non-reverse transcribed C57BL/6 mRNA was PCR amplified (lane "-").

Several factors could contribute to the maintenance of peripheral CD19-positive surface LC-negative cells: *de novo* production of B cells, or proliferation in equilibrium with cell death and/or long life. To determine whether repression of LC completely eliminates *de novo* production of B cells in the bone marrow, IgL-repressible mice were treated with doxycycline for two generations (to exclude transfer of maternal B cells to the fetus), after which B cells in the periphery were sought. Flow cytometry analysis of splenocytes of IgL-repressible mice treated in this way revealed no B cells in the periphery (Figure 3C, right panel). This result indicates that B cells are not produced *de novo* when production of lambda is repressed.

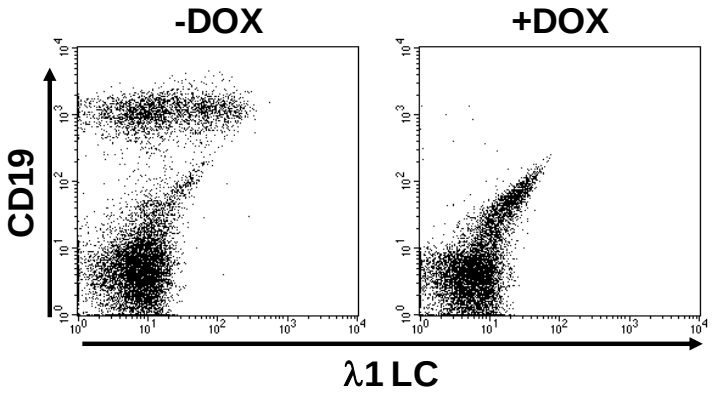

FIGURE 3C: λ LC repression abrogates *de novo* B cell production in the bone marrow of repressible Ig mice. Flow cytometry analysis of splenocytes obtained from IgL repressible mice generated and maintained with (right) or without (left) doxycycline for 2 generations. Splenocytes were stained with rat anti-mouse monoclonal antibodies directed to CD19 or to λ1, λ2 and λ3.

It is possible that B cells surviving repression of LC are the progeny of rare B cells that proliferated to maintain the B cell compartment. To test this idea we analyzed spleen sections co-stained with anti-Ki67 (a cell division marker). Figure 3D shows that no μHC-positive cells express Ki67, and hence CD19-positive B cells were not proliferating to any great extent following repression of LC. Consistent with this concept, tunnel analysis shows that splenocytes of mice treated with doxycycline for 4 weeks were not undergoing apoptosis (as might be expected in rapidly proliferating populations of cells) (Figure 3E). These results show that repression of LC expression did not cause enhanced turnover of B cells, and therefore the maintenance of surface LC–negative B cells must be owed to long life.

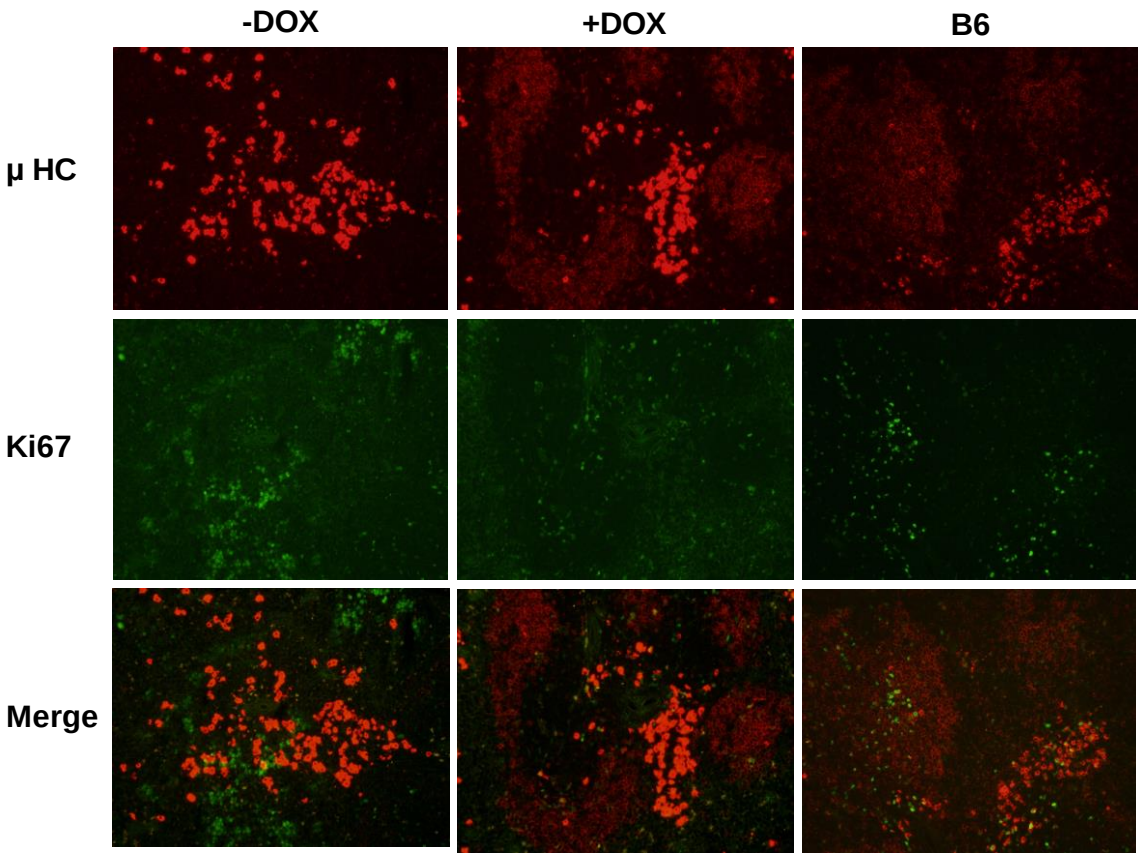

FIGURE 3D: Spleen sections of IgL repressible mice fed (right) or not fed (left) doxycycline and B6 controls were co-stained with anti-Ki67 (FITC-conjugated) and anti-IgM (H+L) antibodies (Rhodamine-conjugated), as noted (100X). Surface Ig-negative B cells do not stain with anti-Ki67 antibody and thus are not dividing.

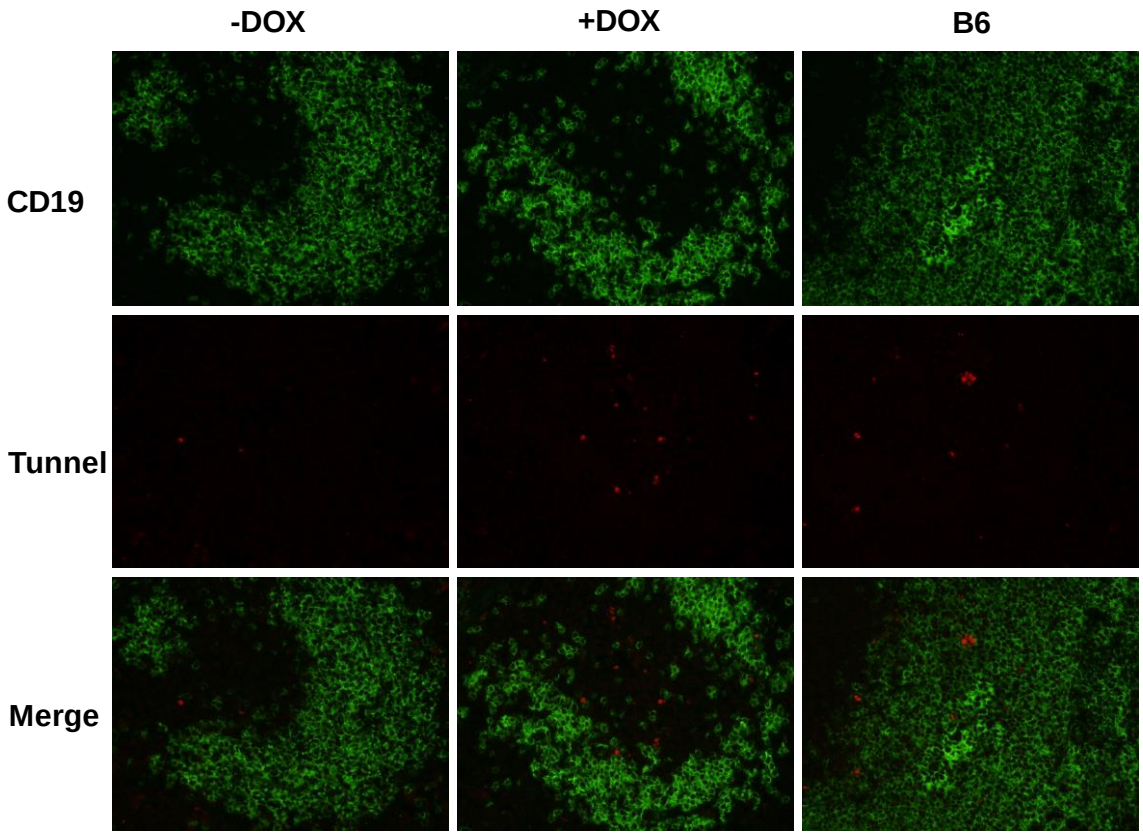

FIGURE 3E: Spleen sections of IgL repressible mice fed (right) or not fed (left) doxycycline as well as B6 controls were co-stained with tunnel antibody (Rhodamine-conjugated) and anti-CD19 antibody (FITC-conjugate d), as noted (100X). λ_1LC-negative B cells are TUNEL-negative and thus not undergoing apoptosis. Results shown are representative of three independent experiments.

HC expressed on the surface of cells is full length and signals

Our results indicate that surviving surface LC-negative B cells continue to produce μHC and suggest the possibility that μHC expression in the absence of LC functions as a receptor. Because unpaired full-length μHCs are thought to be retained in the ER (Mielenz et al., 2003), we asked whether μHC expressed on the surface was full length. Western Blot analysis of cellular and surface μHC separated by SDS-PAGE shows that LC-negative B cells expressed predominantly the full-length μHC on the surface (Figure 4A lane 5). In addition to the full length protein, splenocytes from repressible IgL mice also produced a lower molecular mass band, visible in lanes 3 through 6 of figure 4A, migrating with an approximate molecular mass

of about 50 kDa. The 50 kDa molecular mass band could correspond to IgG or alternatively a truncated HC, as has been described in some B cell malignancies (Witzig and Wahner-Roedler, 2002). Figure 4B shows that the 50 kDa MW bands are not IgG because they fail to be detected with an antibody directed against IgG.

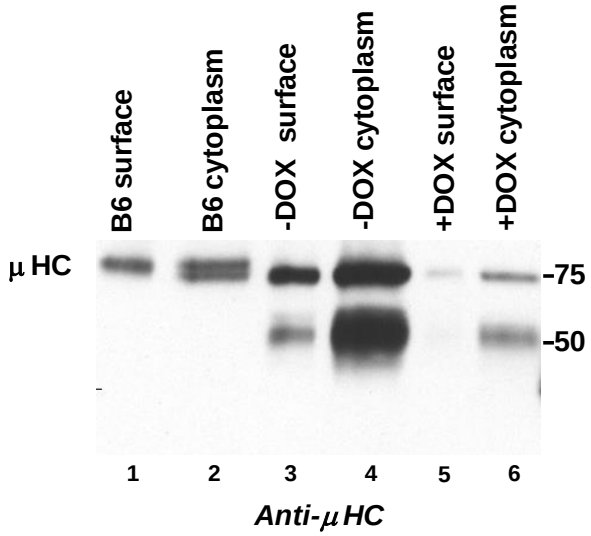

FIGURE 4A: Western Blot analysis of surface and cytoplasm μHC in IgL repressible mice fed (right) or not fed (left) doxycycline as well as B6 controls. Protein lysates obtained from isolated B cells were surface biotinilated and immuno-precipitated (with streptavidin beads). Biotinilated samples (surface) and non biotinilated samples (cytoplasm) were analyzed by 10% SDS PAGE and blotted with goat anti-mouse IgM (H+L) antibody. Figure shows that the full length μHC is expressed on the surface of LC-negative B cells. Approximate molecular mass (MW) is noted on the right.

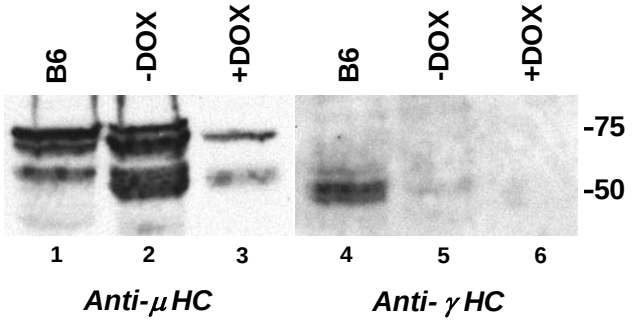

FIGURE 4B: Western Blot analysis of IgG and IgM in IgL repressible mice fed (right) or not fed (left) doxycycline as well as B6 controls. Samples were analyzed by 10% SDS PAGE and blotted . Blotted proteins were revealed with a goat anti-mouse IgM (H+L) or antimouse IgG antibodies as indicated. Western Blot shows

that 50KD MW bands detected by the anti-IgM (H+L) antibody are not IgG because they fail to be detected with an antibody directed against IgG. Approximate molecular mass (MW) is noted on the right.

To determine whether the 50kDa band resulted from a truncated heavy-chain, lacking CH1 or the VH exon, we analyzed HC gene expression by RT-PCR. Figure 4C shows that repressible Ig splenocytes express only the full-length RNA with no evidence of deletions in the variable or constant region exons (Figure 4C) indicating that the 50 kDa molecular mass band apparent in lanes 3-6 of figure 4A, is likely a degradation product. The decrease in LC-repressed splenocytes μHC mRNA, apparent in figure 4C, is owed to the fact that LC-repressed mice have 5-fold to 6-fold fewer B cells compared to LC-expressing mice. Our results indicate the possibility that limited light chain availability renders the monoclonal VH17.2.25 μHC more susceptible to proteolysis compared to the polyclonal μHC population in B6.

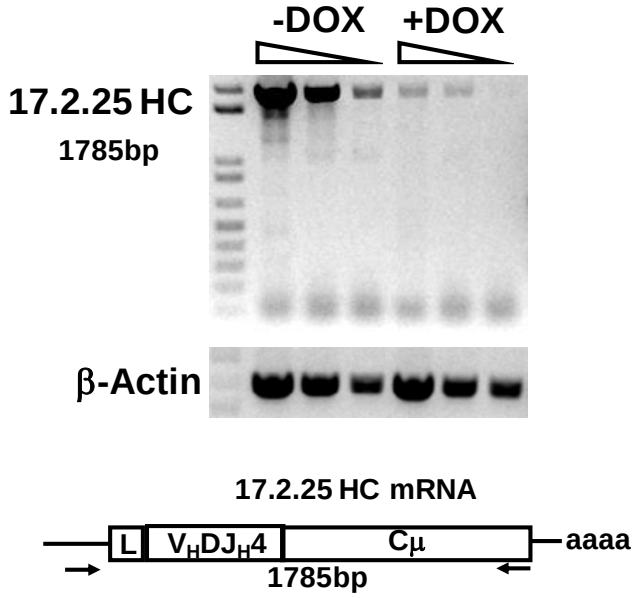

FIGURE 4C: RT-PCR analysis of the length of the μHC mRNA obtained from splenocytes of IgL repressible mice fed or not fed doxycycline, as indicated. Figure shows that the mRNA is full length, i.e., it does not contain deletions in the V or C exons. Below is shown a schematic representation of the RT-PCR primers sites respective to μHC mRNA. 1:1, 1:5 and 1:25 dilutions of the cDNA were used.

The surface expression of unpaired μHC suggested the possibility that HC alone delivers B cell survival signals. To answer this question, we compared changes in the level of intracellular free Ca^{2+} in B cells stimulated with anti-IgM, whole antibody or F(ab)2. Naïve QM B cells and surface LC-positive B cells from repressible IgL mice responded to IgM cross-linking by quickly increasing the intracellular Ca^{2+} (Figure 4D, upper and medium panel, blue line). Changes in the level of intracellular free Ca^{2+} in QM or surface LC-positive B cells stimulated with anti-IgM had similar kinetics as those following stimulation with anti-Igλ LC (green lines). In contrast, surface LC-negative B cells responded to IgM cross-linking with a modest increase in the intracellular Ca^{2+} originating a lower amplitude and somewhat retarded peak when compared to λLC-positive B cells or QM B cells (Figure 4D, lower panel, blue line). The amplitude of the Ca^{2+} peak in response to IgM cross-linking on the surface LC-negative B cells was reduced relative to wild type B cells, possibly owing to decreased surface receptor density. Cross-linking Ig-L on surface LC-negative B cells originated no response (Figure 4D, lower panel, green line). These results indicate that unpaired μHC generates signals.

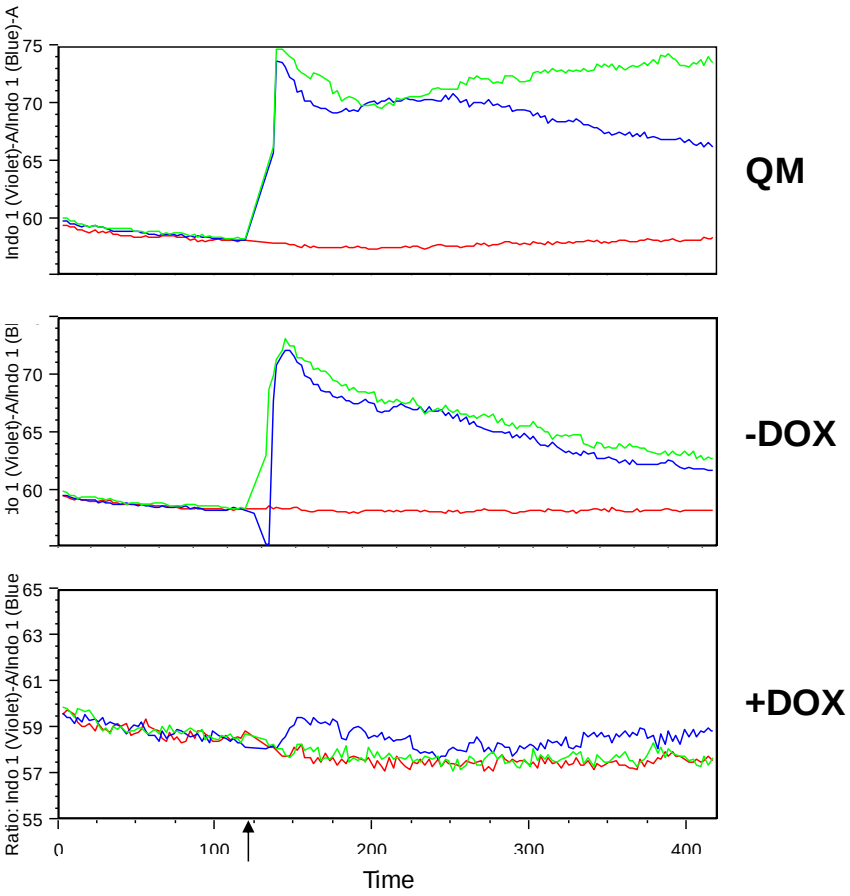

FIGURE 4D: Flow cytometry analysis of Ca^{2+} influx in isolated B cells, obtained from the spleen of QM, or from IgL-repressible mice fed or not fed doxycycline for four weeks. Histograms represent the ratio of violet/blue fluorescence intensities (Indo1 bound to Ca^{2+}/unbound Indo1) over time. The cells were stimulated with either goat anti-mouse IgM (H+L) antibody (blue), with goat anti-mouse lambda antibody (green) or with control antibodies, goat IgG (red) added at 2 minutes after the start (indicated by the arrow). Cross-linking of surface μHC but not λLC causes a modest Ca^{2+} influx by LC-negative B cells (lower panel). Results are representative of four independent experiments.

Unpaired HC in the cytoplasm triggers receptor-independent responses

Because in the absence of LC, HC is retained in the endoplasmic reticulum (ER) by BiP (immunoglobulin HC binding protein) (Vanhove et al., 2001) we investigated whether LC-negative B cells activated a stress response called the unfolded protein response (UPR). We tested activation of several UPR transducers. Activated inositol requiring enzyme endoribonuclease (IRE) (Wu and Kaufman, 2006) excises 26 base pairs from the X-box-

binding protein 1 (XBP-1) mRNA to form XBP-1 spliced (s). Activation of activating transcription factor 6 (ATF6), another UPR transducer, induces the transcription of *xbp-1* and ER chaperone genes; eukaryotic translation initiation factor a, subunit α kinase (PERK) activation transiently inhibits cap-dependent protein synthesis and induces C (EBP) homologous protein (Chop). Figure 5 shows that LC-negative B cells express the spliced (s) and unspliced (u) *xbp-1* messages and have increased levels of *BiP* mRNAs consistent with inositol requiring enzyme 1α and activating transcription factor 6 activation. Chop expression is consistent with PERK activation. These results indicate activation of all three UPR transducers and suggest the possibility that the UPR may contribute to the λLC-negative B cells' long life.

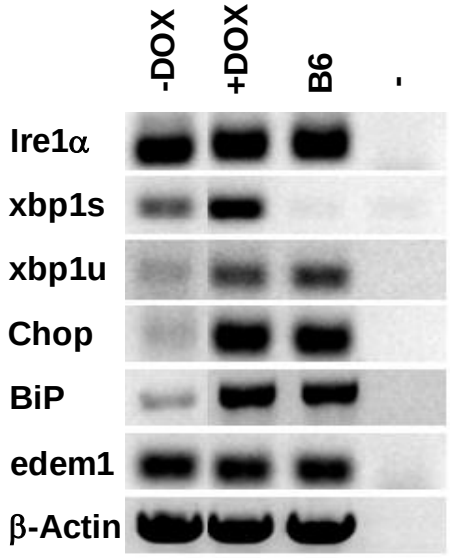

FIGURE 5: RT-PCR analysis of UPR genes. mRNA obtained from CD19-positive B cells isolated by MACS purification from pooled spleens of 6 repressible IgL mice fed or not fed doxycycline for 2 weeks. mRNA from adult C57BL/6 was used as a positive control. To control for DNA contamination of RNA samples, non-reverse transcribed C57BL/6 mRNA was PCR amplified (lane -).

We also tested whether several unfolded protein response gene mRNAs were produced in SL and L chain-negative, Igα-positive Ag8.653 expressing or not the transgenic VH17.2.25 chain. Our results (not shown), indicate that *ire1*, *xbp-1*, spliced and unspliced, *chop*, and *edem1* were equally expressed independently of the transgenic VH17.2.25 chain. These results

indicate that in transformed cells activation of the UPR occurs independently of heavy chain expression. These results do not contradict the possibility that accumulation of HC in the ER triggers the unfolded protein response in mature B cells independently of BCR stimulation.

Discussion

The property of memory that uniquely distinguishes adaptive immunity in host defense requires long-term survival of B cells after first exposure to antigen. How long-term survival is achieved is not completely understood but thought to depend on surface expression of the antigen receptors. Thus, survival of T cells depends on engagement of the TCR with self-MHC plus self-peptide, assuring that only functional T cells live (Anderson et al., 1999). In contrast to T cells, B cells recognize novel structures that are cleared from the organism. Thus, survival of mature B cells is thought to require a mature BCR (Kraus et al., 2004; Kurosaki, 2002; Lam et al., 1997), even though the necessity of a generic self-ligand has not yet been resolved.

As one possibility, BCR may promote B cell survival by signaling constitutively. This idea is supported by the work of Lam et al. (Lam et al., 1997) and Kraus et al. (Kraus et al., 2004), who showed that ablation of HC or Igα signaling in mature B cells led to rapid cell death (in days). However, survival of B cells without BCR is not without precedent since BCR-less B cells expressing an Epstein Barr virus receptor instead of BCR survive *in vitro* and *in vivo* (Casola et al., 2004). Because Epstein Barr receptor expressing B cells appear to activate Tyr-phosphorylation of BCR targets, Casola and collaborators propose that BCR signaling is necessary for the survival of B cells (Casola et al., 2004).

Here we report that survival of mature B cells does not require the complete BCR but rather the mere production of unpaired HC suffices to assure B cell survival. In LC-negative B cells, μHC can be expressed on the surface. Since surface μHC cross-linking induces a modest calcium influx we concluded that μHC can signal. Signaling by unpaired surface μHC may be one mechanism promoting mature B cell survival. Our results showing long-term survival of B cells expressing HC unpaired with LC support the concept that a B cell autonomous mechanism governs B cell longevity in the absence of a complete BCR.

Functional antibodies lacking light chains are produced by B cells in camels (Muyldermans and Lauwereys, 1999), nurse shark and wobbegong shark (Greenberg et al., 1995) and in ratfish (Rast et al., 1998) indicating that in these species B cells develop and persist in the absence of a conventional BCR. Moreover, expression of dromedary HC-only antibodies in the mouse sustains B cell development (Janssens et al., 2006; Zou et al., 2005).

HC-only antibodies in camels possess molecular adaptations, such as the loss of CH1 domain, to avoid interaction with light chains and binding to the heavy chain binding protein (BiP) thus escaping retention in the ER (Conrath et al., 2003; Knarr et al., 1995). Truncated heavy chains have also been associated with disease in mice and in humans causing myeloma (Brandt et al., 1984) or heavy chain disease (Corcos et al., 1995; Corcos et al., 1991; Fermand and Brouet, 1999), respectively. Heavy-chain disease–associated μ proteins lacking the rearranged VDJ exon (ΔμHC) produce unpaired HC receptors that are signaling competent. Corcos et al. found that expression of ΔμHC promotes B cell differentiation in the bone marrow (Corcos et al., 1995; Corcos et al., 1991) and in the periphery. However, mature B cells expressing ΔμHC are larger and have shorter half-lives than wild type B cells (Corcos et al., 2001). This is in spite of the fact that ΔμHCs overcome BiP-mediated ER retention and are expressed on the surface (Corcos et al., 2001). Since the HC produced by the IgL-repressible mouse is not truncated, our results indicate that when the availability of light chain is limited, full-length heavy chains may escape ER trapping and form signaling competent receptors. Those may be important to promote survival of cells that loose LC expression owing to somatic hypermutation or receptor editing.

That unpaired full-length μHC mediates some of the BCR functions was determined by Schuh et al. and Galler et al. (Galler et al., 2004; Schuh et al., 2003) who found that wild type full-length μHC unbound to LC is expressed on the surface, promotes *in vivo* differentiation of pro-B cells , induces IL-7-dependent growth and signals, causing decreased recombination

activating gene expression and allelic exclusion at the HC locus . It is possible that expression of unpaired full-length HC contributes to the survival of human B cells lacking the conventional BCR in normal subjects (Pollok et al., 1987). Expression of unpaired full-length HC may also contribute to the development of B cell malignancies by increasing the probability of survival of cells undergoing illegitimate DNA recombination or extensive DNA breaks.

Expression of a BCR that is signaling competent does not by itself assure long life since immature B cells that are recent bone marrow emigrants have a very short lifespan (days) (Gaudin et al., 2004). Thus, other mechanisms in addition to receptor-generated signaling are necessary. Our results showing expression of unpaired μHC in the cytoplasm of LC-negative B cells suggest the possibility that cytoplasmic μHC contributes to the survival of mature B cells. Perhaps the HC-only cells that survive repression of LC do so because of persistent unfolded protein response (UPR) initiated prior to repression of LC and accumulation of HC in the ER as described following cytokine and LPS stimulation of B cells (Gass et al., 2002; Skalet et al., 2005). However, in contrast to LPS-activated blasts, HC-only cells do generate long lived B cells, suggesting that accumulation of HC in the ER may govern cell survival in addition to inducing terminal differentiation.

Acknowledgements

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THE DOUBLE EDGED SWORD OF AID[1]

Xiaosheng Wu[*,†,¶], **Pedro Geraldes**[*], Jeffrey L. Platt[*,‡,§,¶] and Marilia Cascalho[*,‡,§,¶2]

THE DOUBLE EDGED SWORD OF AID

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Transplantation Biology Program[*], and the Departments of Biochemistry and Molecular Biology[†], Immunology[‡], Pediatrics and Adolescent Medicine[§], and Surgery[¶]

Mayo Clinic, Rochester, Minnesota 55905

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Abstract

Activation-induced cytidine deaminase (AID) is required for immunoglobulin (Ig) class switch recombination, a process that introduces DNA double strand breaks in B cells. We show here that AID associates with DNA-PKcs promoting cell survival, presumably by resolving DNA double strand breaks. Wild type cells expressing AID mutants that fail to associate with DNA-PKcs, or cells deficient in DNA-PKcs or 53BP1 expressing wild type AID, accumulate γH2AX foci, indicative of heightened DNA damage response. Thus AID has two independent functions. AID catalyzes cytidine deamination that originates DNA double strand breaks needed for recombination, and it promotes DNA damage response and cell survival. Our results thus resolve the paradox of how B cells undergoing DNA cytidine deamination and recombination exhibit heightened survival and suggest a mechanism for hyper IgM type II syndrome associated with AID mutants deficient in DNA-PKcs binding.

Introduction

Activation-induced cytidine deaminase (AID) promotes somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin genes (Durandy, 2003a). AID cytidine deaminase activity was first proposed on the basis of its homology with the apoB m RNA editing catalytic polypeptide 1 (APOBEC-1) (Muramatsu et al., 1999). The cytidine deaminase property of AID led to two distinct hypotheses to explain diversification of the immunoglobulin (Ig) genes. The first, the RNA editing hypothesis, proposes that AID, like APOBEC-1, modifies unknown RNA precursors that in turn, originate endonucleases that cleave the DNA encoding the Ig genes. The findings by Begum et al (Begum et al., 2004), showing that AID-dependent DNA cleavage in CSR requires de novo protein synthesis is in agreement with this hypothesis. The second, the DNA deamination hypothesis, proposes that AID deaminates cytidine to uracil directly in the DNA encoding the Ig genes . Compatible with the second hypothesis are the results of Petersen-Marth et al. showing that expression of AID in *Escherichia coli* originates a mutator phenotype that yields nucleotide transitions at dC/dG and the findings of Dickerson et al. (Dickerson et al., 2003), Pham et al. (Pham et al., 2003) and Chaudhuri et al. (Chaudhuri et al., 2003) showing that AID deaminates DNA substrates *in vitro*.

How exactly AID introduces point mutations or executes class switch recombination is not yet understood, but it is generally thought that cytidine deamination of DNA or RNA somehow generates double strand breaks in Ig DNA (Celeste et al., 2002; Durandy, 2003a; Papavasiliou and Schatz, 2000; Petersen et al., 2001; Zhang, 2003). Ordinarily, cells respond to DNA double strand breaks by undergoing cell cycle arrest to allow time for repair (Nyberg et al., 2002) and respond to persistent damage by inducing apoptosis, presumably as a protection against illegitimate recombination (Pfeiffer et al., 2000). However, B cells undergoing Ig class switch do not die, presumably because they efficiently repair DNA double strand breaks. While RAD54 RAD52 and RAD51 repair proteins are needed for AID induced Ig gene

conversion in chicken cell lines (Bezzubova et al., 1997b; Sale et al., 2001) whether AID directly recruits repair factors to the locales of cytidine deamination is not known.

Materials and Methods

Generation of AID wild type and mutant constructs

RNA was obtained from C57BL/6 mouse lymph nodes using Trizol™ reagent. AID cDNA was produced by reverse transcription using oligo-dT primer and amplified by PCR using Turbo *pfu* polymerase (Stratagene) and primer set wu160/wu167. Full-length AID cDNA was cloned in-frame into pUHD10S vector downstream of Flag tag sequences. AID deletion mutants were generated by PCR using Turbo *pfu*. The primer sets used to generate the deletion mutants were: mutant F1: wu160/wu166; mutant F3: wu160/wu165; mutant F4: wu162/wu167; mutant F6: wu161/wu167; and mutant ΔC: wu160/wu174. We used QuickChange mutagenesis kit™ (Stratagene) to generate AID-DN and AID-R112H point mutation constructs with primer sets wu155/wu156 and wu157/wu158, respectively. PCR fragments were flanked by 5' NheI and 3' XbaI sites to allow subsequent cloning into the pUHD10S vector. The Flag-tagged AID fragment (EcoRI/XbaI) was subcloned into the pCI expression vector (Promega) for transient expression and into the pCI-neo expression vector (Promega) for stable transfections. For expression in 70Z/3 cells and in splenocytes, Flag-AID or Flag-AID-ΔC fragments were cloned into pIRES2GFP vector (BD clontech) upstream of IRES-EGFP sequence followed by subcloning of the Flag-AID-(or Flag-AID-ΔC)-IRES2-EGFP cassette into the pMSCV-puro retroviral vector (BD clontech). To produce the GST-AID fusion protein, the full-length AID cDNA was cloned in-frame into the pGEX4T1 vector (Amersham). All sequences were verified by DNA sequencing.

Cell culture, transfection and retroviral transduction

Wild type mouse embryonic fibroblasts (MEF) were generated from C57BL/6 mouse embryos at 14.5 dpc and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 1.0 IU/ml of penicillin G and 0.5 IU/ml of streptomycin. HeLa cells and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% FCS, 1.0 IU/ml of penicillin G and 0.5 IU/ml of streptomycin. DNA-PKcs$^{-/-}$ MEF cells (PK33N) (Araki et al., 1999) were kindly provided by Dr. David J. Chen

(Lawrence Berkeley National Laboratory) and maintained in alpha-MEM supplemented with 10% FCS, 1.0 IU/ml of penicillin G and 0.5 IU/ml of streptomycin. Mouse pre-B lines 70Z/3 and 18.81 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1.0 IU/ml of penicillin G and 0.5 IU/ml of streptomycin. LPS cultures were prepared by incubating B cells (95% purity, isolated with a MACS column), $2x10^5$/well/100µl, with 10µg/ml LPS (Sigma) in RPMI 1640 medium supplemented with 10% FCS, 1.0 IU/ml of penicillin G and 0.5 IU/ml of streptomycin. B cells were obtained from C57BL/6 mice as described (Cascalho et al., 1996).

Transient transfection of AID wild type or mutant construct into HeLa, HEK293 or MEF cells was performed with Lipofectamine (Invitrogen). Expression of wild type AID in 70Z/3 cells and in splenocytes was done by retroviral transduction. Linearized pMSCV- IRES2-EGFP-Puro or pMSCV-Flag-AID-IRES2-EGFP-Puro DNA was stably transfected into RetroPack PT67 (BD Clontech) packaging cells by electroporation, whereas pMSCV-Flag-AID-□C-IRES2-EGFP-Puro was transiently transfected into the same packaging line using lipofectomine. The virus-containing supernatant of each kind (in DMEM medium) was collected every 24 hours and further concentrated by centrifugation at 6000g for 4 hours. Virus pellets were resuspended in 1/50 volume of complete RPMI1640. LPS cultures were transduced with proviruses at the time of seeding in 100 µl of virus-containing supernatant supplemented with 10 µg/ml of LPS. AID and AID-ΔC expressing splenocytes were analyzed with a FACScalibur (Becton Dickinson) by measuring GFP positivity and propidium iodide (PI) stain for cell death.

Subcellular fractionation

Nuclei and cytosol fractions were prepared according to published protocols (Kihlmark and Hallberg, 1998). Briefly, 10^7 HEK293 cells expressing Flag-AID were harvested and washed three times with PBS. Cells were resuspended in 1 ml of lysis buffer [10 mM HEPES, pH7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT and protease inhibitor cocktail (Roche)] on ice for 20 minutes followed by 20 gentle strokes for homogenization with a loose fit Dounce

homogenizer. The homogenate was overlaid on top of 200 ul of 40% sucrose in lysis buffer and centrifuged at 800g for 15 minutes in a centrifuge with swinging bucket rotor. The supernatant (corresponding to the cytosolic fraction) and the pellet (corresponding to the nuclear fraction) were collected and extracted with 10× lysis buffer (Wu et al., 2003).

Immunoprecipitation and Western Blotting

Immunoprecipitation was performed as described previously (Wu et al., 2003). To identify AID-binding proteins, 2x108 HeLa cells expressing Flag-AID were used for a large-scale immunoprecipitation with EZview™ anti-Flag (M2) beads (Sigma). Otherwise, routine immunoprecipitation was carried out by using 2x107 cells expressing Flag-AID. In each experiment, one-fourth of the precipitated proteins (equivalent to 5x106 cells) were resolved on a 6% SDS-PAGE (for DNA-PKcs and Ku80) or 12% SDS-PAGE (for AID). In the experiments testing DNA dependence of AID/DNA-PKcs binding, one-fourth of precipitated beads were incubated with 100 µl of PBS alone or 10 mM of STDDP in PBS for 2 hours on ice to crosslink protein complexes. After quenching crosslinking reaction with 10 µl of 1.0 M Tris-HCl, pH7.4 for 15 minutes and washed twice with PBS, the beads were then treated with 20 units of DNase-I (as indicated in Figure 3B) in a 40 µl of PBS at room temperature for 30 minutes, followed by 2 washes prior to SDS-PAGE analysis under reducing condition (5% β-mercaptoethanol) to cleave crosslinked complexes. Mouse monoclonal anti-Flag (M2) antibody was purchased from Sigma. Rabbit anti-DNA-PKcs (specific for human DNA-PKcs, SC-9051), monoclonal anti-Ku80 (SC-5280) were purchased from Santa Cruz. Monoclonal anti-DNA-PKcs (specific for mouse DNA-PKcs; NA57) was obtained from Oncogene™, goat anti-GST antibody is a product of Amersham (27-4577-01), monoclonal anti-β tubulin was obtained from Santa Cruz Biotechnology, Inc. (SC-5274) and rabbit anti-AID serum was a generous gift from Dr. Frederick W. Alt (Harvard Medical School).

DNA-PKcs in vitro pulldown by GST-AID

Recombinant GST and GST-AID fusion protein were purified from bacterial DL21 cells. 500

ng of GST or GST-AID fusion protein beads were incubated with 0.5 ml cell extracts obtained from 2×10^7 HEK293 cells overnight at 4°C, in the presence or absence of 2 μg of single stranded (ss) DNA, 2 μg of double stranded (ds) DNA, or 20 units of DNase-I. ssDNA was a 59-base oligonucleotide containing RGYW repeats (AGCTGGCAGGCTAGCAAGTTGGTT-GGCAAGCAGGTAAGCAGG CAAGCTGGCTGAATTCC) (Chaudhuri et al., 2003). dsDNA was an EcoRV linearized pBluescript KS vector (Stratagene). Beads were washed and analyzed as described above.

Immunofluorescence staining and microscopy

Immunofluorescence staining and confocal microscopy were performed essentially as described previously (Wu et al., 2003). For microtubule staining in HeLa cells expressing Flag-AID, cells were fixed with methanol at -20°C for 10 minutes, air dried, rehydrated, repermeabilized with 0.05% Triton X-100 in PBS for 3 minutes and blocked for 90 minutes with blocking buffer (5% normal goat serum, 1% glycerol, 0.1% BSA, 0.1% fish skin gelatin, 0.04% sodium azide) followed by staining with anti-β tubulin antibody (Sigma, T5293). Cytoplasmic protein extraction was done by incubating HeLa cells expressing GFP or Flag-AID with 50 μg/ml of digitonin in PBS on ice for 5 minutes followed by 4 washes with PBS and fixation with 4% paraformaldehyde**.** Affinity purified rabbit anti-γH2AX were kindly provided by Dr. Junjie Chen (Mayo Clinic, Rochester, MN). Cell death of mouse embryonic fibroblasts was determined by TUNEL assay (Promega) and by the presence of condensed chromatin or fragmented nuclei in DAPI staining (Shimodaira et al., 2003).

Identification of AID-associated proteins

The proteins co-immunoprecipitated with Flag-AID from HeLa cells were resolved on a SDS-polyacrylamide gel electrophoresis (PAGE) (4 to 15% polyacrylamide gradient) (Bio-Rad) and stained with Coomassie G-250 (Bio-Red). Protein bands were excised and analyzed using Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry by the Rockefeller University Protein Resource Center (New York, NY).

Oligonucleotides used

Wu155:CTTCGCAACAAGTCTGGCTGCCGCGTGCAATTGTTGTTCCTACGCTACATC

Wu156:
GATGTAGCGTAGGAACAACAATTGCACGCGGCAGCCAGACTTGTTGCGAAG

Wu157: CAGCCTGAGGATTTTCACCGCGCACCTCTACTTCTGTGAAGACCGC

Wu158: GCGGTCTTCACAGAAGTAGAGGTGCGCGGTGAAAATCCTCAGGCT

Wu160: GAATCAGCTAGCGACAGCCTTCTGATGAAGCAAAAG

Wu161: GAATCAGCTAGCGGCTGCCACGTGGAATTGTTGTTC

Wu162: GAATCAGCTAGCGAGGGGCTGCGGAGACTGCACC

Wu165: GAATCATCTAGATTAAGGCTCAGCCTTGCGGTCTTCAC

Wu166: GAATCATCTAGATTAATTTTCTACAAATGTATTCCAGCAG

Wu167: GAATCATCTAGATTAAAATCCCAACATACGAAATGCATC

Wu174: GAATCATCTAGATTAGTCATCGACTTCGTACAAGGGCAAAAGG

Results

AID binds to DNA-PKcs in the nucleus

We considered the possibility that association of AID with cofactors could promote survival of cells undergoing immunoglobulin gene diversification. To determine whether AID associates with other molecules, we analyzed AID protein complexes obtained from HeLa cells expressing AID. AID was immunoprecipitated from lysates, and the identity of any co-precipitates was sought by mass spectrometry. Figure 1A shows a Coomassie-stained gel image showing proteins co-immunoprecipitated with AID. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry identified the largest co-precipitated protein as DNA-PKcs with a molecular weight of 486 kDa based on 34 matched peptides. Other proteins identified were heat shock 70kDa protein 8 isoform 1 (HSC70) (12 matched peptides), tubulin beta 2 (15 matched peptides) and a protein similar to mitochondrial solute carrier family 25 (4 matched peptides) (Figure 1).

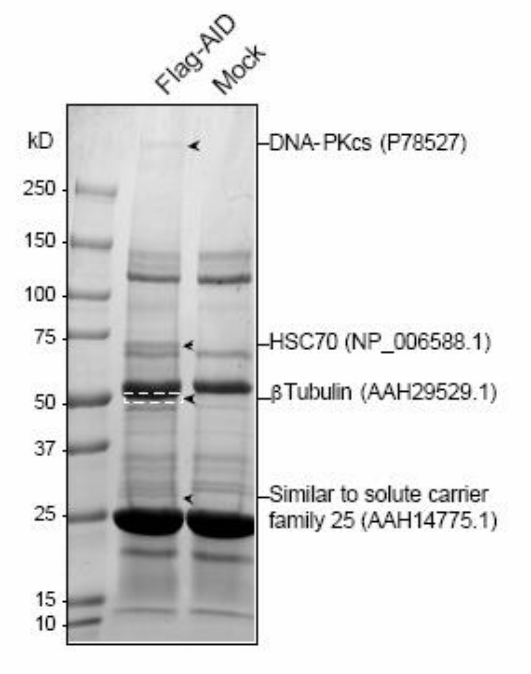

FIGURE 1: Identification of AID binding proteins. Comassie Blue staining of 4-15% gradient SDS-PAGE analysis of anti-Flag immunoprecipitates of lysates obtained from HeLa cells transiently transfected with Flag-

AID or empty vector (mock). Flag-AID co-migrates with the antibody light chain (25KD). AID binding proteins were identified by MALTI-TOF mass spectrometry. Protein identities and accession numbers are indicated.

Since DNA-PKcs is required to efficiently resolve by non-homologous end-joining the DNA double strand breaks associated with class switch recombination (Manis et al., 2002), we questioned whether AID associated with DNA-PKcs in B lineage cells and cells other than HeLa. Figure 2A shows DNA-PKcs co-immunoprecipitated with AID and vice versa, in extracts from HeLa cells, human embryonic kidney 293 (HEK293) cells, and murine B cells (70Z/3) transfected with Flag tagged AID. We further show in Figure 2B that endogenously expressed AID in 18.81 B cells (Bachl et al., 2001) binds to DNA-PKcs (right panel). Our results indicate that the association of AID with DNA-PKcs may reflect the function of AID in B cells.

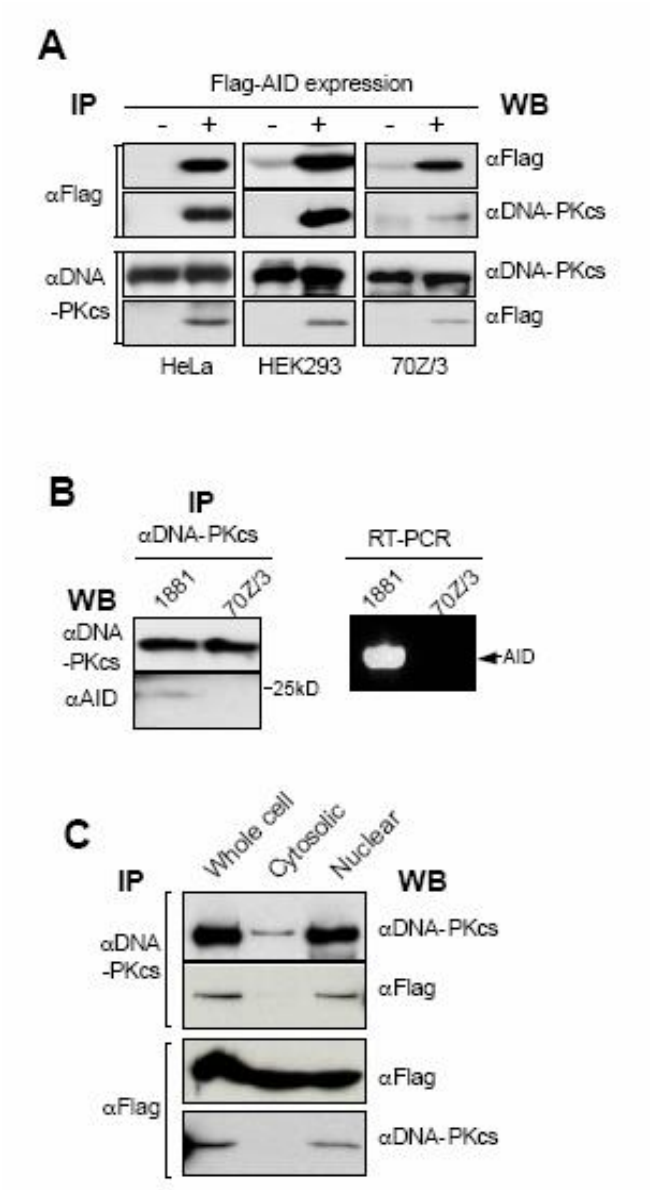

FIGURE 2: AID associates with DNA-PKcs of B and non-B cells. (A) AID co-precipitates with DNA-PKcs in various AID transfected cell lines. Shown are western blot (WB) analysis of anti-Flag or anti-DNA-PKcs immunoprecipitates (IP) of cell lysates obtained from HeLa, HEK293 or 70Z/3 cells transfected with Flag-AID (+) or empty vector (-). Blots were probed with either anti-Flag (αFlag) or anti-DNA-PKcs (αDNA-PKcs) antibodies, as indicated. Mouse Ig light chain co-migrating with 25 kD Flag-AID, is seen as a weak band in the controls. Figure is representative of 3 independent experiments. **(B)** Endogenous AID associates with DNA-PKcs. Shown is a western blot (WB) analysis (left panel) of anti-DNA-PKcs immunoprecipitates (IP) of cell lysates from mouse pre-B cell line 18.81 that constitutively expresses AID or from mouse pre-B cell line 70Z/3 that does not express endogenous AID. Blots were probed with either anti-DNA-PKcs antibodies or rabbit polyclonal anti-AID (Chaudhuri et al., 2003) as indicated. Expression of endogenous AID in 18.81 and 70Z lines

was verified by RT-PCR as shown in the right panel. **(C)** AID and DNA-PKcs associate in the nucleus. Shown is a western blot analysis of Flag-AID or DNA-PKcs immunoprecipitates from whole cell extracts, cytoplasmic fraction, or nuclear fraction obtained from HEK293 cells expressing AID. The blots were probed with either anti-DNA-PKcs or anti-Flag antibodies, as indicated.

AID is mainly localized to the cytoplasm (Rada et al., 2002), whereas DNA-PKcs localizes predominantly in the nucleus (Koike et al., 1999), and yet they associate with one another. We asked whether the association of DNA-PKcs and AID took place in the nucleus where presumably AID and DNA-PKcs function. Figure 2C shows that DNA-PKcs co-precipitates with AID in the nuclear fraction but not in the cytoplasmic fraction of cell extracts (Figure 2C). Our results indicate that the association of DNA-PKcs and AID takes place in the nucleus, representing the small fraction of total cellular AID (Figure 2C).

Association of AID and DNA-PKcs requires the C-terminal and deamination domains of AID

Because DNA-PKcs is required for the repair of DNA double strand breaks during class switch recombination (Manis et al., 2002) but is dispensable for somatic hypermutation (Bemark et al., 2000), our findings showing that AID associates with DNA-PKcs predict that the complexes mediating class switch recombination and somatic hypermutation are distinct. Recent findings from two laboratories support this idea (Barreto et al., 2003; Ta et al., 2003). Barreto et al. (Barreto et al., 2003) found that an AID mutant that lacks the C-terminal 10 amino acids retained cytidine deaminase activity but failed to promote class switch recombination. Ta et al. (Ta et al., 2003) found that some subjects with type II hyper-IgM syndrome have mutations in AID causing truncation or disruption of the C-terminal domain. These individuals have a severe defect in class switch but normal somatic hypermutation (Ta et al., 2003). These findings suggested that the C-terminal region of AID is necessary for class switch recombination in B cells (Barreto et al., 2003; Ta et al., 2003).

We questioned whether the selective defect in class switch recombination of AID C-terminus deletion mutants reflected defective association with DNA-PKcs. To test this idea, we generated a series of AID deletion mutants and tested the mutants for association with DNA-PKcs. C-terminal truncation mutants lost [mutant F1 (positions 1-154) and ΔC (positions 1-189)] or markedly reduced [mutant F3 (positions 1-123)] their ability to bind to DNA-PKcs (Figures 3A and 3B), suggesting that the C-terminal domain of AID is necessary for the formation of AID/DNA-PKcs complexes. On the other hand, mutant F6 (positions 54-198) lacking the N-terminal 53 amino acid segment of AID retained the ability to bind to DNA-PKcs. However, mutant F4 (positions 124-198) with a larger deletion encompassing the deamination domain did not bind to DNA-PKcs (Figures 3A and 3B). These results led us to speculate that the binding of AID to DNA-PKcs also requires the deamination domain.

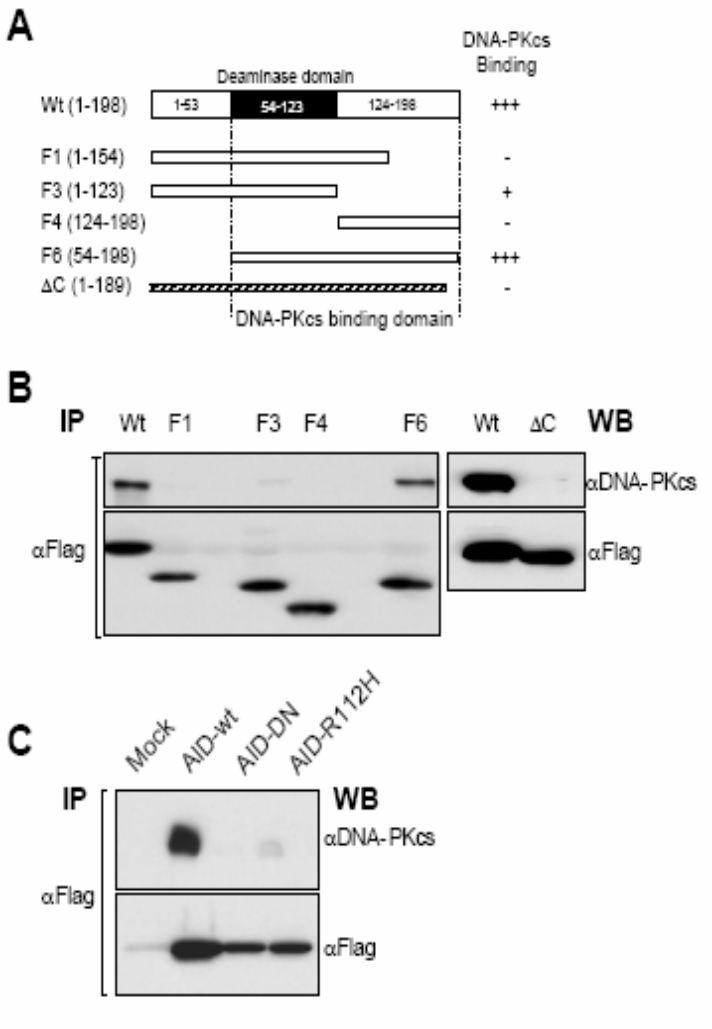

FIGURE 3: AID deamination and C-terminal domains are required for binding to DNA-PKcs. (A) Schematic domain representation of full-length AID and deletion mutants. DNA-PKcs binding of wild type or deletion mutants is noted on the right. Wild type AID, Mutants F1 and F6 associate with DNA-PKcs; Mutants F1, F4 and ΔC do not. (B) Deamination and C-terminal domains of AID are necessary for binding to DNA-PKcs. Shown is a western blot (WB) analysis of anti-Flag immunoprecipitates (IP) obtained from lysates of HEK293 cells transiently transfected with wild type (wt) or deletion mutants (F1, F3. F4, F6 and ΔC) of AID. Blots were probed with either anti-Flag or anti-DNA-PKcs antibodies, as indicated. (C) Cytidine deamination defective AID mutants do not bind to DNA-PKcs. Shown is a western blot (WB) analysis of anti-Flag immunoprecipitates obtained from lysates of HEK293 cells stably expressing Flag tagged AID-wt, dominant negative H56R/E58Q mutant (DN), or R112H mutant AID. Blots were probed with either anti-Flag or anti-DNA-PKcs antibody, as indicated. The figures are representative of 3 independent experiments each.

Deletion of the AID deamination domain could abrogate binding to DNA-PKcs because of alteration in the conformation of the AID C-terminus or because of inactivation of the cytidine deaminase activity. To determine whether inactivation of cytidine deaminase activity abrogated binding to DNA-PKcs, we tested the ability of two deamination defective AID mutants to associate with DNA-PKcs. An AID dominant negative mutant (AID-DN, H56R/E58Q) (Chaudhuri et al., 2003; Papavasiliou and Schatz, 2002) exhibited no binding, and an AID variant found in some patients with type II hyper-IgM syndrome (AID-R112H) (Revy et al., 2000; Ta et al., 2003) exhibited very little binding to DNA-PKcs (Figure 3C). These results show that subtle mutations in the deamination domain of AID that impair cytidine deaminase activity abrogate binding to DNA-PKcs.

Association of AID and DNA-PKcs is DNA-dependent

Some propose that AID promotes isotype class switch by deaminating cytidines in the DNA of switch regions (Reina-San-Martin et al., 2003), others that AID edits RNA originating a class switch specific factor such as an exonuclease or endonuclease to resect DNA ends (Doi et al., 2003). Since our results indicate that the AID deamination domain is necessary for the recruitment of DNA-PKcs, we asked whether DNA is a co-factor for AID and DNA-PKcs complex formation. To answer this question, we tested whether a GST-AID fusion protein

associates with DNA-PKcs in the presence or in the absence of DNA. Figure 4A shows that addition of exogenous single stranded (ss) DNA or double stranded (ds) DNA increases the efficiency of DNA-PKcs precipitation from HEK293 extracts by immobilized GST-AID (Figure 4A, lanes 3 and 4). Precipitation reflected the specific properties of DNA since adding DNase-I disrupted GST-AID/DNA-PKcs complex formation (Figure 4A, lanes 5-7). Likewise, DNase-I treatment also caused dissociation of co-immunoprecipitated AID/DNA-PKcs complexes (Figure 4B, left panel). However, when the immunoprecipitates were crosslinked with primary amine-reactive and thiol-cleavable crosslinker 3, 3'-Dithiobis-(sulfosuccinimidylpropionate) (STDDP) that only crosslinks proteins, DNase I treatment could no longer dissociate DNA-PKcs from AID (Figure 4B, right panel). These results indicate that AID and DNA-PKcs form a stable complex through protein-protein interaction requiring DNA as a co-factor.

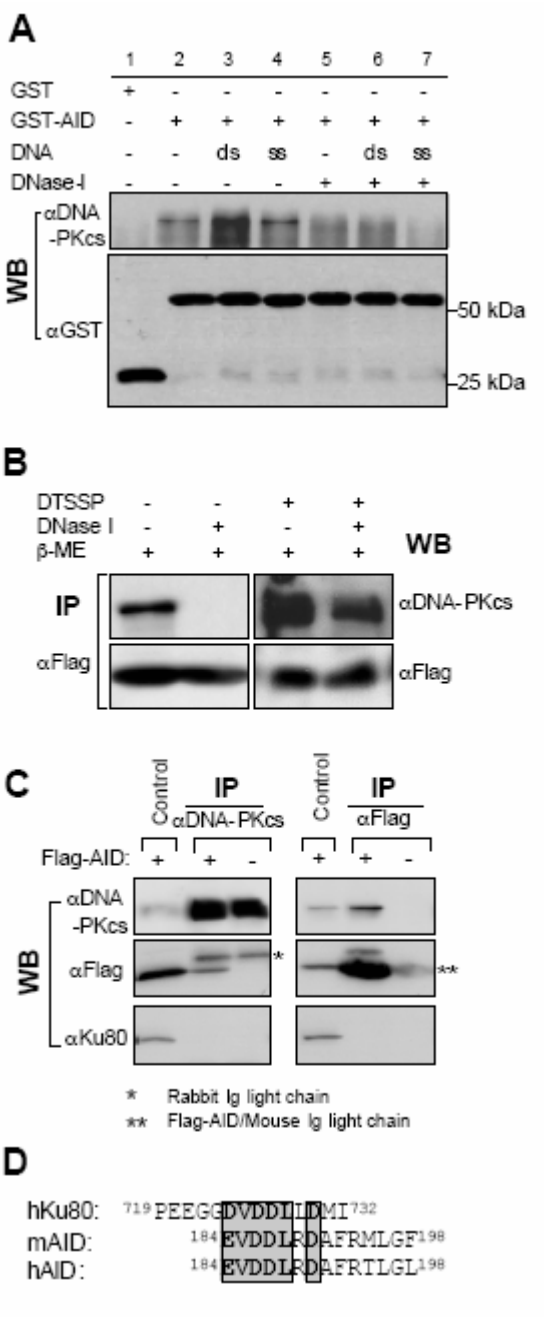

FIGURE 4: AID associates directly with DNA-PKcs and association requires DNA as a co-factor. (A) DNA is a co-factor for DNA-PKcs binding to AID because the complex is dissociated by DNase I treatment (lanes 5, 6 and 7) and enhanced by addition of double (lane 3) and single stranded DNA (lane 4). Shown is a western blot (WB) analysis of GST or GST-AID bead pulldowns of HEK293 whole cell extracts in the presence or absence of single stranded (ss) or double stranded (ds) DNA and with or without of DNase-I. Blots were probed with either anti-DNA-PKcs or anti-GST antibodies, as indicated. (B) DNA-PKcs and AID associate *via* protein-protein interaction because cross linking abolishes sensitivity of the complex to DNAase I. Shown is a western blot (WB) analysis of anti-Flag immunoprecipitates obtained from whole cell lysates of HEK293 cells expressing

Flag tagged wild type AID treated with or without a cross linker (DTSSP) and subsequently with or without 20 units of DNase-I after immunoprecipitation. Cross linking was disrupted with β-mercaptoethanol before SDS-PAGE and western blot analysis. Blots were probed with either anti-Flag or anti-DNA-PKcs antibodies, as indicated. **(C)** DNA-PKcs association with AID is independent of Ku80. Shown is a western blot (WB) analysis of immunoprecipitated AID/DNA-PKcs complexes probed with anti-DNA-PKcs, anti-Flag or anti-Ku80 antibody, as indicated. To show the presence of the tested proteins and the antibody reactivity, we included total cell extract from AID expressing HEK293 cells (first lane in each panel) equivalent to 1% of material used in immunoprecipitation as positive control. **(D)** Sequence alignments of the C-terminal domains of mouse (m), human (h) AID and human (h)Ku80.

It is generally thought that high affinity binding of DNA-PKcs to DNA breaks requires the association with Ku70/Ku80 heterodimer (West et al., 1998). Hence, we asked whether Ku80 was also present in AID/DNA-PKcs complexes. Figure 4C shows that Ku80 is not detectable in the AID/DNA-PKcs complexes while it is clearly present in the cell extracts (Figure 4C, control lanes). This result suggests that DNA-PKcs does not bind to Ku80 while it is associated with AID.

AID C-terminal deletion mutant causes cell death

How does the association of DNA-PKcs contribute to the function of AID? DNA-PKcs is thought to contribute to the generation of lymphocyte receptors by promoting the repair of double strand breaks generated during V(D)J and class switch recombination by NHEJ (Gao et al., 1998). Because persistent DNA double strand breaks cause cell death, repair mediated by DNA-PKcs may be critical for cell survival. Consistent with this idea is the finding that mice deficient in DNA-PKcs lack B and T lymphocytes (Gao et al., 1998) and also exhibit hypersensitivity to agents that cause double strand breaks such as ionizing radiation (Gao et al., 1998). Hence, we asked whether recruitment of DNA-PKcs by AID promotes survival of cells undergoing DNA breaks associated with cytidine deamination (Petersen et al., 2001). To test this idea, we measured DNA damage foci and death of cells expressing the wild type or the C-terminal deletion mutant AID (AID-ΔC). Figure 5A shows that transient expression of AID-ΔC that does not bind DNA-PKcs in mouse embryonic fibroblasts (MEF) resulted in

18% dead cells, while transient expression of wild type AID led to only 5% dead cells, which was comparable to the cell death observed in non-transfected cells (NT) (4.5%). Our results indicate that recruitment of DNA-PKcs by AID promotes cell survival.

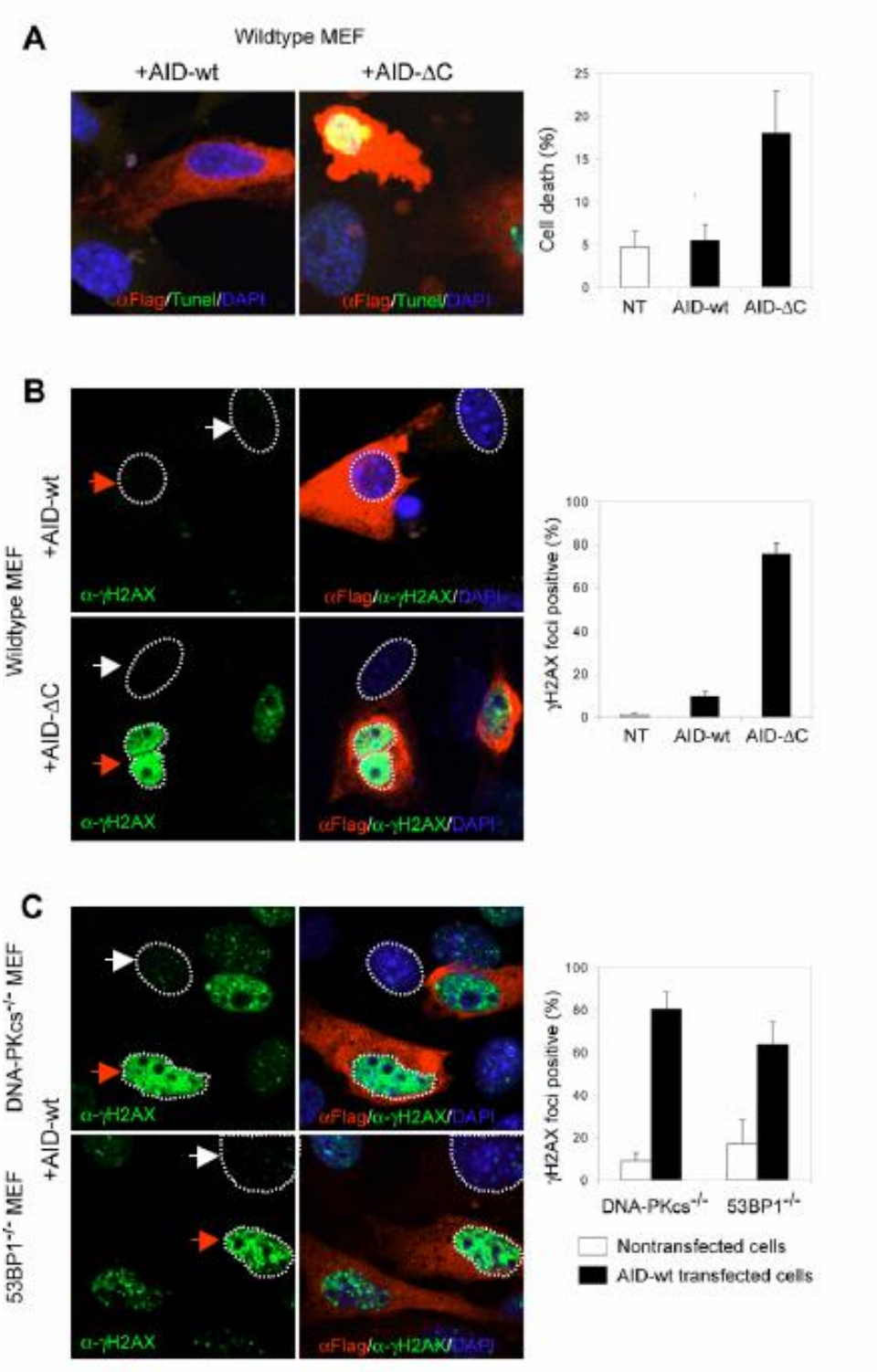

FIGURE 5: Association of AID with DNA-PKcs protects cells from death and do not form nuclear γH2AX foci. (A) Expression of an AID C-terminal deletion mutant (AID-ΔC) but not wild type AID (AID-wt) induces cell death. Shown are representative confocal images (left panels) of wild type MEF cells transiently transfected with Flag tagged AID-wt or Flag tagged AID-ΔC. At 24 hour post-transfection, apoptosis of Flag positive cells (red) was scored for TUNEL positivity (green) and DAPI staining (blue). The chart (right panel) shows the proportion of apoptotic cells 24 hours following transfection. Data was collected from 4 independent experiments by scoring at least 500 cells per experiment. NT represents non-transfected cells. (B) Expression of C-terminal deleted mutant AID (AID-ΔC) but not wild type AID (AID-wt) induces the formation of nuclear γH2AX foci. Shown are representative confocal images (left panels) of wild type MEF cells transiently transfected with Flag-AID-wt or Flag-AID-ΔC, immunostained with anti-Flag (red), anti-γH2AX (green) antibodies and counterstained with DAPI (blue). The chart (right panel) shows the proportion of nontransfected (left panel, white arrows) or transfected (red arrows) cells with large nuclear γH2AX foci 24 hours following transfection. Data was collected from 3 independent experiments by scoring at least 150 cells per experiment. (C) Expression of wild type AID causes DNA damage foci in repair deficient cells. Shown are representative confocal images (left panels) of DNA-PKcs $^{-/-}$ or 53BP1$^{-/-}$ MEF cells transiently transfected with Flag-AID-wt, immunostained with anti-Flag (red), anti-γH2AX (green) antibodies and counterstained with DAPI (blue). The chart (right panel) shows the proportion of nontransfected (left panels, white arrows) or transfected (red arrows) cells with large nuclear γH2AX foci 24 hours following transfection. Data was obtained from 3 independent experiments by scoring at least 150 cells per experiment.

AID C-terminal deletion mutant causes heightened DNA damage response

To test whether expression of AID-ΔC led to DNA damage response, we stained MEF cells expressing wild type or AID-ΔC proteins for phosphorylated H2A histone family member X (γH2AX), which binds to DNA double strand breaks forming foci (Petersen et al., 2001). Figure 5B shows that only 1.1% of non-transfected MEF cells (Figure 5B, white arrows) and 9.4% cells transfected with AID-wt (Figure 5B, white arrows) exhibited detectable nuclear γH2AX foci. In contrast, 75.6% MEF cells expressing AID-ΔC had massive accumulation of nuclear γH2AX foci (Figure 5B, red arrows). Our data is consistent with the concept that recruitment of DNA-PKcs by AID is needed to resolve DNA double stranded breaks.

DNA-PKcs and 53BP1 are necessary to resolve AID-generated γH2AX foci

Absence of γH2AX nuclear foci in wild type MEF cells expressing wild type AID could result from prompt DNA repair or alternatively from decreased DNA break formation. To determine the contribution of DNA repair to the lack of DNA damage foci in cells expressing wild type AID, we examined DNA damage foci in repair-deficient cells expressing wild type AID. Figure 5C shows that expression of wild type AID induced nuclear γH2AX foci in 80.5% DNA-PKcs$^{-/-}$ cells, while only 9.3% of non-transfected cells scored positive. Similarly, expression of wild type AID also induced γH2AX foci in 64% 53BP1$^{-/-}$ cells (Ward et al., 2003) while only 17.2% non-transfected cells were positive. These results indicate that AID generates DNA double strand breaks and γH2AX foci, which accumulate in the absence of DNA-PKcs or other DNA repair components such as 53BP1.

AID C-terminal deletion mutant impairs survival of activated B cells

Our results indicating that AID deficient in DNA-PKcs binding causes accumulation of DNA double strand breaks suggest a mechanism for the selective class switching defect in some patients with hyper IgM type II syndrome. We tested whether expression of AID mutants deficient in DNA-PKcs binding owing to C-terminal deletion (AID-ΔC) impaired survival of B cells undergoing class switch recombination. To do this, we transduced lipopolysaccharide (LPS) activated B cells obtained from spleens of wild type C57BL/6 mice with retroviral vectors encoding wild type AID (AID-wt), or AID-ΔC or GFP (Figure 6A). Figure 6B shows that while 91.6% AID-ΔC transduced B cells (GFP-positive) were dead at day 3 of LPS culture, only 25.2% of the cells transduced with AID-wt and 19.2% of the cells expressing only GFP died (Figure 6B). Our results indicate that AID-ΔC causes death of cells undergoing LPS stimulation. We observed that the surviving AID-ΔC expressing cells did not class switch, thus confirming the findings of Barreto et al. (Barreto et al., 2003) who showed that AID-ΔC does not promote class switch. Because AID-ΔC transduced B cells undergo cell death, our results indicate that AID-ΔC associated defective class switch is owed to the death of the cells undergoing class switch recombination.

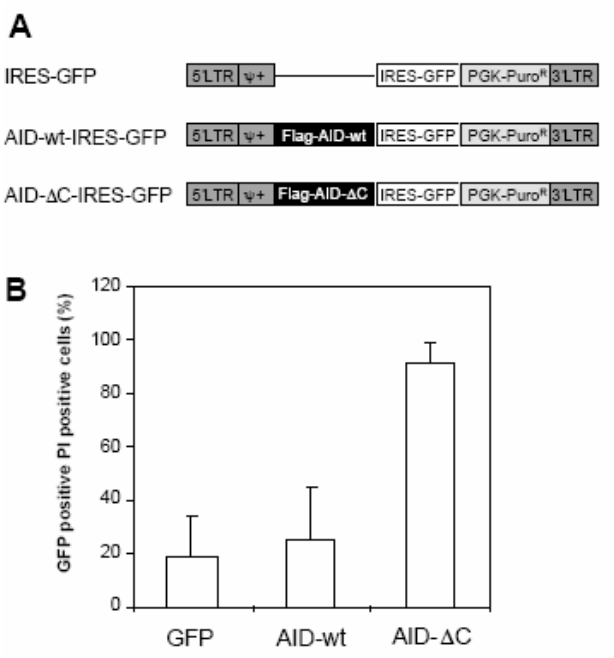

FIGURE 6: AID C-terminal deletion mutant (AID-ΔC) induces cell death of LPS stimulated B cells. (A). Schematic representation of the retroviral constructs used to transduce B cells. (B) C-terminal deleted mutant AID (AID-ΔC) but not wild type AID (AID-wt) induces death of LPS stimulated B cells. Cell death was scored by propidium iodide (PI) staining of LPS stimulated splenocytes transduced with proviruses encoding green fluorescent protein (GFP), wild type AID (AID-wt), or C-terminal deleted AID mutant (AID-ΔC), at day 3. PI positive cells were identified by flow cytometry analysis. Graph represents the fraction of transduced B cells (GFP-positive) that were PI-positive (y-axis). Data was collected from 3 independent experiments and represents means and standard deviations.

C-terminal deletion does not alter AID intracellular localization

McBride et al. (McBride et al., 2004) and Ito et al. (Ito et al., 2004) proposed an alternative mechanism for the impaired class switch recombination by AID-ΔC mutants. These authors suggested that deficient class-switch is a consequence of deletion of a leucine rich nuclear export signal causing predominant nuclear localization of AID-ΔC (Ito et al., 2004; McBride et al., 2004). However, Figures 5A and 5B show that Flag-tagged AID-ΔC like wild type AID, localized predominantly in the cytoplasm of mouse embryonic fibroblasts. Our results showing that removal of the nuclear export signal (aa 189-198) in the Flag-tagged AID-ΔC did

not alter intracellular localization of the protein indicate that the pro-death effect of AID-ΔC is not due to nuclear accumulation.

Intracellular localization of AID may not be primarily governed by nucleocytoplasmic shuttling. If AID shuttled between cytoplasm and nucleus, then addition of nuclear localization signal should drive AID to the nucleus. Figures 7A-E show that while addition of three consecutive copies of SV40 nuclear localization signals (Moore and Blobel, 1992) efficiently drives GFP in to the nucleus (Figure 7B), AID remains in the cytoplasm (Figure 7E). These results suggest the possibility that AID is actively retained in the cytoplasm. Consistent with this possibility, cytoplasmic localization of wild type AID is resistant to digitonin treatment (Figure 7F) that effectively depletes GFP from the cytoplasm of the cells (Figure 7C). Our data indicates that AID is retained in the cytoplasm by digitonin-resistant cytoskeletal elements. One of such elements is β-tubulin because β-tubulin specifically co-precipitated (Figures 1 and 7G) and partially co-localized (Figures 7H-J) with AID.

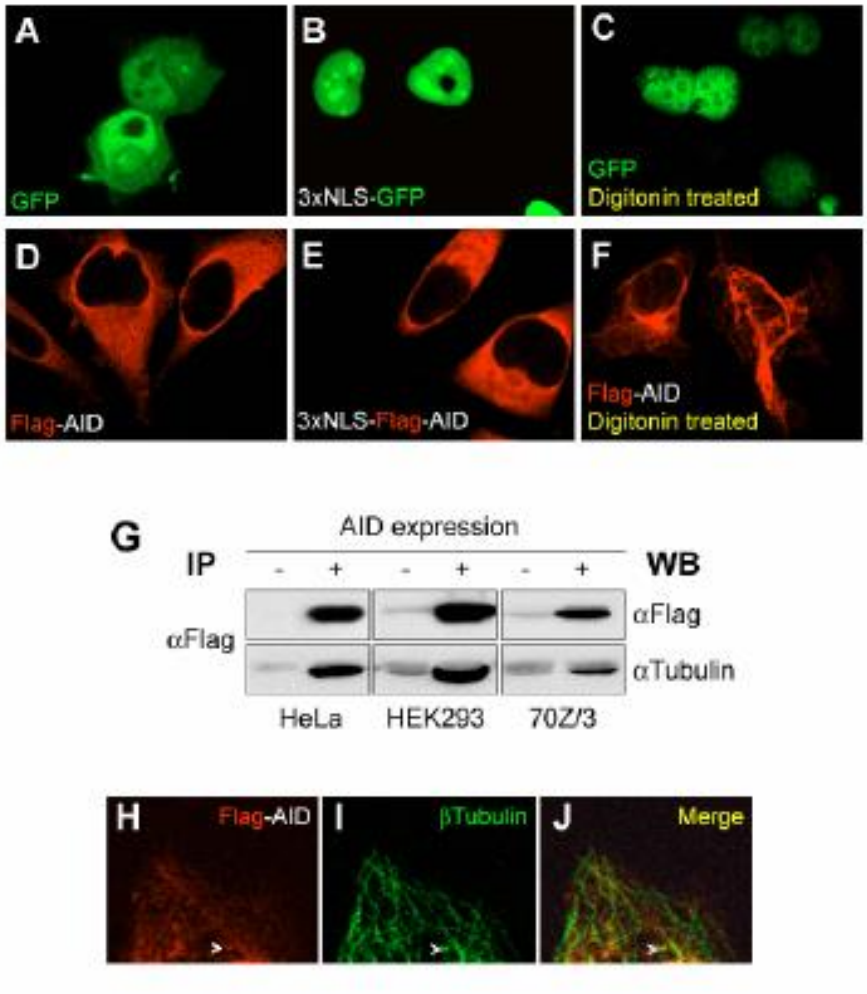

FIGURE 7: AID is retained in the cytoplasm by cytoskeletal proteins. GFP distributes in the cytoplasm and in the nucleus of HeLa cells (A) while Flag-AID localizes predominantly to the cytoplasm. Addition of three SV40 nuclear localization signals to GFP (3xNLS-GFP) or to Flag-tagged AID (3xNLS-AID) causes nuclear localization of GFP (B) but not Flag-tagged AID (E). Digitonin treatment of cells removes GFP (C) but not Flag-tagged AID (F) from the cytoplasm indicating cytoplasmic retention of AID. (G) AID associates with β-tubulin. Shown are western blot (WB) analysis of anti-Flag immunoprecipitates (IP) of cell lysates obtained from HeLa, HEK293 or 70Z/3 cells transfected with Flag-AID (+) or empty vector (-). Blots were probed with either anti-Flag or anti-β tubulin antibodies, as indicated. Mouse Ig heavy chain migrating slight above β tubulin, is seen as a weak band in the controls. (H-J) AID partially colocalized with β tubulin. Shown are confocal images of HeLa cells expressing Flag-AID double stained with Flag (red)and anti-β-tubulin (green) antibodies. Arrowheads show co-localization of AID and β tubulin.

It is possible that the properties of AID-GFP fusion proteins studied by McBride et al. (McBride et al., 2004) and by Ito et al. (Ito et al., 2004) differ from the properties of Flag-tagged AID or of the native AID. While the intracellular distribution of Flag-tagged AID-ΔC is determined by cytoplasmic retention, the distribution of AID-ΔC-GFP fusions is not. Instead, intracellular distribution of AID-ΔC-GFP fusion proteins is determined by nucleo-cytoplasminic shuttling.

Discussion

We show here that AID promotes cell survival by recruiting DNA-PKcs to the DNA, hence resolving double strand breaks. Our findings explain how B cells survive while undergoing DNA double strand breaks during class switch recombination. Consistent with this notion, compromised class switch recombination is a common phenotype in mice deficient in proteins that are necessary for DNA damage repair such as H2AX (Reina-San-Martin et al., 2003), ATM (Pan-Hammarstrom et al., 2003), 53BP1 (Manis et al., 2004) and Mre11 (Lahdesmaki et al., 2004).

Gell and Jackson (Gell and Jackson, 1999) showed that DNA-PKcs associates with Ku70/Ku80 binding to the 12 amino acid C-terminal tail of the Ku80 subunit. Our data show that DNA-PKcs binding to AID requires the C-terminus of AID (Figures 3A and 3B). Such a striking binding parallel made us wonder whether the C-terminal domain of Ku80 might be homologous in any way to the C-terminal domain of AID. Sequence alignment of the 14 amino acid C-terminal domains of AID and Ku80 revealed that both sequences possess a common (E/D)VDDL(X)D motif (Figure 4D). The common motif in the C-terminal domains of AID and Ku80 suggest competition for the same binding site on DNA-PKcs. This mechanism may coordinate the formation of AID/DNA-PKcs and of Ku70/Ku80/DNA-PKcs complexes for repair of double strand breaks by non-homologous end joining (NHEJ) following cytidine deamination of DNA.

We propose a working model to explain how AID promotes survival of cells undergoing DNA double strand breaks. AID binding to DNA through the DNA deamination domain undergoes a conformational change resulting in the exposure of the C-terminal domain that, in turn, recruits DNA-PKcs to the DNA. Upon the generation of DNA breaks by cytidine deamination, DNA-PKcs initiates NHEJ by dissociating from AID and re-associating with Ku80 to assemble the NHEJ repair complex. Our results thus explain previous observations

by Barreto et al. (Barreto et al., 2003) and Ta et al. (Ta et al., 2003) who showed that C-terminal deleted AID mutants fail to promote class switch recombination. Our results suggest that the mechanism underlying hyper IgM type II syndrome associated with AID mutations that truncate the C-terminal portion of the molecule are owed to selective death of B cells stimulated to undergo class switch recombination because of accumulation of DNA double strand breaks. That C-terminal deleted AID mutants may sustain somatic hypermutation (Ta et al., 2003) is also in agreement with our findings since somatic hypermutation is less dependent on the recruitment of DNA-PKcs than class switch recombination, possibly due to engagement of alternative repair mechanisms (Bardwell et al., 2004; Cascalho et al., 1998). Our results may also explain why lack of DNA-PKcs causes class switch deficiency for all isotypes except IgG1 (Manis et al., 2002), while the SCID mutation, which truncates DNA-PKcs, allows moderate class switching to all isotypes (Bosma et al., 2002). While AID in the absence of DNA-PKcs is unable to recruit repair factors to the sites of cytidine deamination, it may do so in the presence of DNA-PKcs with the SCID mutation.

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Final discussion and conclusion

This thesis originated from studies on the molecular mechanisms that contribute to B cell survival before and after activation in response to immunologic stimuli. We examined which BCR properties impart long life to B cells and asked if expression of unpaired immunoglobulin heavy chain promotes the survival of B cells. Next, we examined the molecular mechanisms of B cell survival in the presence of DNA double strand breaks during terminal differentiation, namely, the role of AID in DNA repair during terminal differentiation.

To exist, B cells must express a functional receptor on the surface. How does the B cell receptor contribute survival signals to the cell is not known. The research related in this thesis examined the contribution of unpaired immunoglobulin heavy chain to the survival of B cells following repression of immunoglobulin light chain expression. We studied the fate of B cells in a murine model where expression of light chain can be conditionally repressed without impairing heavy chain production (Geraldes et al., 2007). We found that survival of mature B cells did not require a completed B cell receptor but rather the mere production of unpaired heavy chain sufficed to assure B cell survival. Immunoglobulin light chain-negative B cells expressed μHC on the surface and the μHC retained signalling competency since cross-linking induced calcium influx. Generally, expression of unpaired heavy chain on the surface of B cells requires overcoming aggregation in the endoplasmic reticulum, a function dependent on the second exon of the μ constant region and mediated by association with light chain or by loss of exon 2 as in some B cell malignancies(Fermand and Brouet, 1999). Since we found no heavy chain truncations our results indicate that when the availability of light chain is limited, full-length heavy chains may escape endoplasmic reticulum trapping and form signalling competent receptors (Geraldes et al., 2007).

Signalling by unpaired surface μ heavy chain may not be the only mechanism promoting mature B cell survival. Thus, we addressed if other mechanisms in addition to receptor-generated signalling could be involved. Our results showing expression of unpaired μ heavy chain in the cytoplasm of light chain-negative B cells suggest the possibility that cytoplasmic μ heavy-chain also contributes to the survival of mature B cells through unfolded protein responses (UPR) (Geraldes et al., 2007). We found that heavy chain-only cells do generate long lived B cells, and differentiate into heavy chain producing cells(Geraldes et al., 2007).

Our results showing long-term survival of B cells expressing HC unpaired with LC support the concept that a B cell autonomous mechanism independent of BCR and BCR specificity governs B cell longevity. Since our publication, research by Zhou et al. (Zhou et al., 2013) confirmed our conclusion that B cells expressing unpaired immunoglobulin heavy chain survive. These authors showed that termination of light chain expression by inducible deletion of an Igκ enhancer not only is compatible with B cell survival but promotes recombination activation gene expression and rearrangement of the lambda light chain locus. Since loss of light chain is often a consequence of Ig somatic hypermutation, signalling by unpaired immunoglobulin heavy chain might be essential to the development of tolerance by receptor editing.

The findings related in this thesis identifying the contribution of unpaired immunoglobulin heavy chain to enhance the survival of mature B cells is of relevance for the development of B cell neoplasms. Although B cell neoplasms that express only immunoglobulin heavy-chains are rare (heavy chain-disease) (Fermand and Brouet, 1999) the contribution of unpaired heavy chains to malignancy may extend to B cell lymphomas in which there is an imbalance between Ig heavy chain and light chain expression. Recent work by Jardin et al. (Jardin et al., 2013) identified excess of μ-heavy chain as an independent risk factor for poor prognosis and response to therapy in diffuse large B cell lymphoma, which is the most common type of non-Hodgkin lymphoma.

Activation of B cells is accompanied by profound chromatin changes that include multiple DNA double strand breaks. DNA breaks often cause cells to die and yet B cells not only survive but those that do so acquire long lives in the process. Indeed, most of the cells that contribute to memory responses which can live several decades, have undergone somatic hypermutation and immunoglobulin class switch recombination which cannot be completed without DNA breaks and recombination. To address the question of how B cells survive DNA double strand breaks during terminal differentiation, we examined the role of activation-induced cytidine deaminase (AID), the enzyme that initiates somatic hypermutation (SHM) and class switch recombination (CSR) of Ig genes in DNA repair thus rescuing B cells from DNA damage induced apoptosis. Our studies demonstrated that AID recruits DNA PKcs suggesting that AID effectively coordinates repair in addition to promoting DNA breaks. Consistent with this notion, compromised class switch recombination is a common phenotype in mice deficient in proteins that are necessary for DNA damage repair(Matthews et al., 2014; Zahn et al., 2014).

We proposed a working model to explain how AID promotes survival of cells undergoing DNA double strand breaks. AID binding to DNA through the DNA deamination domain undergoes a conformational change resulting in the exposure of the C-terminal domain that, in turn, recruits DNA-Protein kinase, catalytic sub-unit (PKcs) to the DNA. Upon the generation of DNA breaks by cytidine deamination, DNA-PKcs initiates non-homologous end joining by dissociating from AID and re-associating with Ku80 to assemble the non-homologous end joining repair complex. Our results suggest that the mechanism underlying hyper IgM type II syndrome associated with AID mutations that truncate the C-terminal portion of the molecule are owed to selective death of B cells stimulated to undergo class switch recombination because of accumulation of DNA double strand breaks. That C-terminal deleted AID mutants may sustain somatic hypermutation (Ta et al., 2003) is also in agreement with our findings since somatic hypermutation is less dependent on the recruitment of DNA-PKcs than class switch recombination, possibly due to engagement of alternative repair mechanisms (Bardwell et al., 2004; Cascalho et al., 1998). Our results may also explain why lack of DNA-PKcs

causes class switch deficiency for all isotypes except IgG1 (Manis et al., 2002), while the Severe combined immune-deficiency mutation, which truncates DNA-PKcs, allows moderate class switching to all isotypes (Bosma et al., 2002). While AID in the absence of DNA-PKcs is unable to recruit repair factors to the sites of cytidine deamination, it may do so in the presence of DNA-PKcs with the SCID mutation. The role of DNA-PK in determining the fate of AID induced lesions has been subject of a recent publications. Zahn et al. (Zahn et al., 2014) showed that deletions in the AID C-terminal preclude the DNA repair response necessary to resolve the AID induced DNA lesions and promote Ig class switch recombination, in part by interacting with Ku70, a DNA-PK co-factor. These results confirm our finding showing that DNAPK and Ku70/Ku80 heterodimers are recruited by AID to promote non-homologous end-joining and class switch recombination (Wu et al., 2005). Singh et al. (Singh et al., 2013) extended these concepts by showing that exclusion of DNA-PK, by interaction with RNA export factor germinal centre associated nuclear protein, from AID-induced lesions, steers resolution of DNA breaks towards homologous recombination and gene conversion in chicken-derived DT40 cells.

Our research revealed for the first time a mechanism linking introduction of mutations and B cell survival. Other mechanisms have been suggested and are the subject of a recent review by (Matthews et al., 2014). Chen et al. (Chen et al., 2014, 2015) found that germinal center B cells were much less likely to develop genomic instability than B cells activated in vitro by cytokines in spite of similar targeting by AID. The research suggests that the B cell environment defines susceptibility to genomic lesions caused by Ig diversification.

In summary, my thesis work revealed for the first time that unpaired heavy chain promotes B cell survival on its own, contributing a mechanism that may be of import in the establishment of tolerance or its failure and contributing to neoplastic transformation of B cells. The second manuscript introduced the new concept that the success of the Ig mutator (AID) to generate Ig diversification depends on its intrinsic ability to recruit certain DNA repair pathways to the

lesions created assuring diversification rather than repair with return to the germline configuration.

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